MEMBRANE-MEDIATED CYTOTOXICITY

Benjamin Bonavida and R. John Collier, Organizers

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Plenary Sessions

Poster Sessions

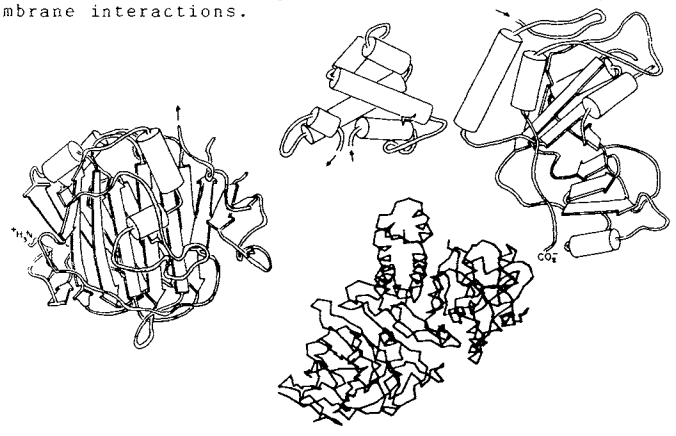
Cytotoxic Proteins: Structure and Activity

G1 CHANNELS FORMED IN LIPID BILAYER MEMBRANES BY DIPHTHERIA, TETANUS, AND BOTULINUM TOXINS, Alan Finkelstein, Department of Physiology & Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461

Diphtheria, tetanus, and botulinum toxins all consist of single polypeptide chains, which, when exposed to mild proteolysis, are cleaved into two-chain molecules in which the heavy-chain C-terminal polypeptide is linked by a disulfide bond to the light-chain N-terminal polypeptide. The light chain of diphtheria toxin is an enzyme, and it has been inferred that the light chains of tetanus and botulinum toxins also have enzymatic activity. For all three toxins, the two-chain structure is the active form, and in the case of diphtheria toxin it has been shown that cell intoxication is a consequence of entry of the enzymatic light chain into the cytosol from an acidic vesicle compartment (the toxin having reached there via receptor-mediated endocytosis). An important unresolved question is how do the heavy chains of these toxins facilitate the entry of the light chains into the cytosol from the acidic vesicle compartment.

We have found that the heavy chains of all three toxins form voltage-dependent channels, with very similar properties, in planar bilayer membranes. Channels are opened by cis positive voltages (the cis side is that side to which protein was added) and closed by cis negative voltages. Channel formation is very pH dependent, and is particularly pronounced when the cis side is at low pH ($\approx$ 4.7) and the trans side is at neutral pH ($\approx$ 7.4). These are the same pH conditions existing across the vesicular membrane of acidic vesicles and the same conditions required for entry of light chains into the cytosol. We have also shown that the channels formed by the three toxins are large, and could accomodate an extended polypeptide chain. Thus, these channels could serve as "tunnel proteins" for the translocation of the active, N-terminal light chains across acidic vesicle membranes.

G2 STRUCTURE AND MECHANISM OF EXOTOXIN A OF PSEUDOMONAS AERUGINOSA, David B. McKay, Viloya S. Allured and Barbara J. Brandhuber, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309-0215. Exotoxin A of P. aeruginosa is a secreted bacterial toxin capable of translocating a catalytic domain into mammalian cells and inhibiting protein synthesis by ADP-ribosylation of cellular elongation factor 2. The protein is a single polypeptide chain of 613 amino acids. We have solved the x-ray crystallographic structure of exotoxin A at 3.0 Å resolution. The molecule has three distinct structural domains; the carboxy terminal domain, comprising approximately one-third of the molecule, has been identified as the enzymatic domain. The other two domains are presumably involved in receptor binding and membrane translocation, although somewhat surprisingly, neither has a substantial region of predominantly hydrophobic amino acids, the normal diagnostic for a domain of a protein being involved in membrane interactions.



G3 MUTANT CULTURED CELLS USED TO STUDY THE ACTIVITY OF ADP-RIBOSYLATING TOXINS. Thomas J. Moehring and Joan M. Moehring, Department of Medical Microbiology, University of Vermont, Burlington, VT 05405

Diphtheria toxin and Pseudomonas exotoxin A ADP-ribosylate protein synthesis elongation factor 2 (EF-2). The site of toxin action is the unique amino acid, diphthamide (2-[3-carboxyamido)-3-(trimethylammonio)propyl]histidine) (1). Two types of mutant cultured Chinese hamster ovary cells were used to study the steps in, and requirements for, post-translational conversion of histidine to diphthamide. One type is altered in the structural gene for EF-2 (codominant). Another type, of which we recognize three complementation groups, is altered in genes that encode the enzymes responsible for post-translational synthesis of diphthamide (recessive). One toxin-resistant form of EF-2, isolated from a recessive mutant, was converted to toxin sensitivity *in vitro*. Conversion occurred in two steps. One step involved addition of methyl groups from S-adenosylmethionine to the modified histidine residue and converted the EF-2 to an ADP-ribosylatable form. The methyltransferase responsible for this modification is present in species as phylogenetically diverse as those of basidiomycetes and primates. A second energy dependent step increased the efficiency of ADP-ribosylation and may be a mechanism for regulating EF-2 activity *in vivo*. Supported by USPHS Grant AI 09100.

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G4 ENTRY MECHANISMS OF PROTEIN TOXINS, Sjur Olsnes and Kirsten Sandvig, Norsk Hydro's Institute for Cancer Research, Montebello, Oslo 3, Norway.

Protein toxins like diphtheria toxin, abrin, ricin, modeccin, shigella toxin and others exert their effect on components of the protein synthesis machinery. The toxins consist of two functionally different parts, one of which binds the toxins to structures at the cell surface, while the other part, which is enzymically active, penetrates the membrane to act on targets in the cytosol. All toxins in this group appear to pass the membrane of intracellular vesicles. In some cases (diphtheria toxin and modeccin) low pH is required for entry to occur, while this is not the case with ricin and abrin which may enter from neutral vesicles. The entry mechanism of diphtheria toxin is known in greatest detail. When the toxin is bound to its receptor at the cell surface, entry directly through the plasma membrane can be induced by exposing the cells to low pH. For entry to occur under these conditions it is required that there is a pH-gradent across the membrane and that permeant anions are present in the medium. When diphtheria toxin is exposed to low pH, a hydrophobic domain in the B-fragment is exposed and may insert itself into the plasma membrane. This insertion strongly inhibits anion antiport in Vero cells. The anion antiporter is regulated by the pH in the cytosol as well as by tumor promoters and growth factors. The relationship between the anion antiporter and the toxin receptor will be discussed.

Receptor-Mediated Endocytosis and Toxin Entry Mechanisms

G5 CHARACTERIZATION OF FOUR GENES REQUIRED FOR THE EXPRESSION OF LDL RECEPTOR ACTIVITY, Monty Krieger, Department of Biology and the Whitaker College, MIT, Cambridge, MA 02139

Biochemical, immunological, and genetic techniques were used to study the defects in four types of LDL receptor-deficient mutant hamster cells (ldlA, ldlB, ldlC, ldlD). ldlA mutants have defects in the structural gene for the LDL receptor and are analogous to cells from patients with familial hypercholesterolemia. Nucleic acid and immunological probes have identified several different mutant forms of the LDL receptor in ldlA cells. These mutant alleles should be useful for structure-function studies of the LDL receptor.

ldlB, ldlC, and ldlD mutants have defects in the Golgi-associated posttranslational processing of LDL receptors and other glycoconjugates. These defects affect the processing of N-linked, O-linked, and lipid-linked carbohydrate chains. Primary and secondary transfectants of ldlB cells have been isolated after treatment of ldlB cells with human DNA. The transfected cells show wild type levels of LDL receptor activity and normal patterns of glycoprotein processing. These results suggest that all of the abnormal phenotypes of ldlB cells are due to defects in a single gene.

The defect in ldlD cells has been identified as a marked deficiency in the enzyme UDP-glucose 4-epimerase. This enzyme normally catalyzes the reversible isomerization of UDP-glucose to UDP-galactose and of UDP-N-acetylglucosamine to UDP-N-acetylgalactosamine. Human deficiency in this epimerase can lead to a severe form of galactosemia. When epimerase-deficient ldlD cells are grown on glucose as the sole sugar source, they cannot synthesize enough UDP-galactose and UDP-N-acetylgalactosamine to allow synthesis of normal N-linked, O-linked and lipid-linked carbohydrate chains. The abnormal glycosylation patterns and the lack of LDL receptor activity in ldlD cells can be completely reversed by providing the cells with exogenous sources of galactose and N-acetylgalactosamine. These sources can include the pure sugars, glycoproteins containing these sugars or other cells grown in close proximity (transfer via intercellular junctions). The results suggest that O-linked carbohydrate chains play an important role in LDL receptor function or stability.

G6 CHO CELL MUTANTS WITH TEMPERATURE-SENSITIVE DEFECTS IMPAIRING BOTH ENDOSOMAL AND GOLGI FUNCTIONS, April R. Robbins and Calvin F. Roff, GBB, NIADDK, Bethesda, Md. 20892.

Three CHO cell mutants exhibiting temperature-sensitive (ts) pleiotropic defects in receptor-mediated endocytosis are distributed between the two complementation groups (designated End1 and 2) found with our non-conditional mutants (1). On shifting End1 and 2 ts mutants to the non-permissive temperature we observed increased resistance to diphtheria toxin, Pseudomonas exotoxin and modeccin, increased sensitivity to ricin, loss of accumulation of Fe from transferrin, loss of uptake via the Man 6-P and α_2-macroglobulin receptors, over-secretion of newly synthesized acid hydrolases and decreased sialylation of some (predominantly secreted) glycoproteins. Although no differences were seen between the most severely affected End1 and 2 mutants with respect to in vivo activities, loss of endocytosis in End1 mutants correlated with loss of ATP-dependent acidification of isolated endosomes, whereas in End2 mutants it did not (collaboration with Renata Fuchs and Ira Mellman).

On shifting the ts mutants to the non-permissive temperature loss of the various activities was quite synchronous; the most sensitive systems began to respond within 0.5h of the shift, the least sensitive within 2h; maximal alteration of all activities was obtained within 4h. In contrast, on return of the End1 ts mutant to the permissive temperature, rates of recovery of activity differed markedly from system to system. Restoration of sensitivity to diphtheria toxin and accumulation of Fe from transferrin (indicative of recovery of endosomal acidification) began immediately and was complete within 5 h, but recovery of Man 6-P and α_2-macroglobulin uptake was not initiated for several hours and was not complete until 24h. Oversecretion of newly synthesized acid hydrolases ceased within 1h of return to the permissive temperature; normal sialylation was restored by 6h, consistent with recovery of Golgi/GERL function. But compartmentalization of newly synthesized β-glucuronidase in lysosomes was delayed for many hours. We conclude that loss of endosomal acidification is followed by loss of some function required for translocation of ligands to lysosomes; recovery of this unidentified function is much slower than recovery of acidification.

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G7 VESICLES INVOLVED IN RECEPTOR MEDIATED ENDOCYTOSIS, Nancy L. Kedersha, Robert P. Searles, Diane F. Hill and Leonard H. Rome, Department of Biological Chemistry and the Mental Retardation Research Center, UCLA School of Medicine, Los Angeles, CA 90024

We have been studying packaging of lysosomal enzymes and lysosomally-directed ligands as a model for understanding how macromolecules are transported in the cell. We previously demonstrated that coated vesicles from rat liver and calf brain are enriched in mannose 6-phosphate receptors (1). These vesicles appear to be involved in transporting newly-synthesized lysosomal enzymes to lysosomes (2). Recently we applied cell fractionation techniques to separate bulk isolated coated vesicles into functionally defined sub-populations and to isolated endosomes containing internalized asialoglycoproteins and asialoglycoprotein receptors as well as mannose 6-phosphate receptors. Coated vesicles can be fractionated into distinct subpopulations on the basis of their overall external charge utilizing preparative agarose gel electrophoresis (3). These vesicles have strikingly similar morphology and coat structure but differ in their a) precise protein compositions b) amount of glycoproteins and c) receptor contents. Labelling of liver endocytic coated vesicles with ^{125}I-asialoorosomucoid reveals that the slower migrating coated vesicles contain the bulk of the endocytosed ligand. A marker for exocytic coated vesicles, acetylcholine esterase, colocalizes with the faster-migrating coated vesicles. The results indicate that preparative agarose gel electrophoresis can be used to separate endocytic from exocytic coated vesicles.

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G8 RECEPTOR-MEDIATED ENDOCYTOSIS: MULTIPLE PATHWAYS FOR LIGAND AND RECEPTOR, Robert J. Fallon, Charles F. Simmons and Alan L. Schwartz, Dana Farber Cancer Institute and Harvard Medical School, Boston, MA 02115

Many hormones, growth factors, and other macromolecular ligands gain entry to intracellular compartments by a process known as receptor-mediated endocytosis (RME). Binding of ligand molecules to specific receptors on the cell surface leads to internalization of receptor-ligand complexes from specialized regions of plasma membrane called coated pits, followed by formation of coated vesicles and uncoated endocytotic sorting structures (endosome; CURL). Sorting of the contents and membrane-bound components of this compartment allows selective targetting of receptors and ligands to their ultimate intracellular destinations. The receptor-mediated endocytosis of galactose-terminal (asialo) glycoproteins into the human hepatoma cell line HepG2 is a well-characterized system which allows one to examine the biochemistry and kinetics of ligand and receptor pathways. The major ligand pathway is that of lysosomal delivery: 50% of an internalized cohort of ligand molecules are sorted from receptor within the acidic environment of CURL vesicles and directed to the lysosome. The receptors recycle back to the cell surface with a mean time of 8 min. Since the receptor lifetime is 30h, a single receptor molecule may internalize ligand and recycle 250 times. A minor route for ligand is termed the ligand-recycling pathway: up to 50% of an internalized cohort of ligand molecules can recycle back to the cell surface, either as free ligand or as ligand-receptor complexes. This process occurs through two biochemically distinguishable pathways with a t1/2 24-84 min. Ligand recycling proceeds independent of the acidity of the sorting compartments. The cellular distribution of receptors and hence capacity for RME can be modulated by exposure to ligand, lysosomotropic amines, and phorbol esters. Exposure of cells to ligand causes a steady state redistribution of receptor and thus a "down regulation" of the surface receptor number. Exposure to amines, however, induces both receptor loss from the cell surface as well as receptor sequestration within a single intracellular compartment. Phorbol esters induce a decrease in cell surface receptor affinity followed by net receptor loss from the cell surface and redistribution to an intracellular compartment. Increased phosphorylation of the receptor accompanies this effect. Furthermore, phorbol esters result in an inhibition of the ligand-recycling pathway, in contrast to the negligible effects of the lysosomotropic amines. Thus, multiple pathways of ligand and receptor movement exist in this system of receptor-mediated endocytosis. Similar strategies will further dissect the mechanisms which regulate ligand and receptor traffic within the cell. Finally, a broader understanding of these intracellular pathways will allow a more refined approach to the targetting of ligand molecules to specific intracellular compartments.

Mechanisms of Viral Attachment and Entry

G9 SITE-SPECIFIC MUTAGENESIS OF THE FUSION PEPTIDE OF INFLUENZA VIRUS HAEMAGGLUTININ, Mary-Jane Gething*#, Robert W. Doms+, and Judy White+, *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724 and + Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510.

The reactions that mediate fusion by enveloped animal viruses are catalysed by specific viral membrane proteins (1). Of these the best characterized is the haemagglutinin (HA) of influenza virus. HA, which is the major glycoprotein of the virion, undergoes a low-pH induced conformational change that initiates the fusion of the virus envelope with endosomal membranes (2-4). Two genetic approaches are currently being employed to analyze the mechanism of HA-mediated fusion and to identify particular amino acids in HA that are directly involved in the fusion process. The first approach involves analysis of the HAs from variant viruses that induce fusion with raised pH thresholds (5-7). The second approach, which will be illustrated in this presentation, involves the use of oligonucleotide-directed, site-specific mutagenesis of a cloned HA gene to alter the nucleotide sequence coding for selected residues of the fusion peptide in the amino terminus of the HA2 subunit. Expression of the mutant HA genes in simian cells has confirmed the central role of the fusion peptide and provided insights into the fusion mechanism (8).

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G10 ENTRY MECHANISMS OF INFLUENZA AND SEMLIKI FOREST VIRUSES, Ari Helenius, Robert Doms, Judy White and Margaret Kielian. Department of Cell Biology, Yale School of Medicine, New Haven, CT 06510.

The spike glycoproteins of orthomyxo-, toga-, rhabdo- and several other virus families have membrane fusion activities triggered by mildly acidic pH (1). This activity is responsible for penetration of the virus from endocytic vacuoles after uptake by host cells. In the case of Influenza hemagglutinin (HA) and Semliki Forest virus spike proteins, where information is most complete, exposure to low pH causes irreversible conformational changes which profoundly modify the structural and functional properties of these well characterized membrane proteins.

The HA, a homotrimer, exposes a hydrophobic moiety in the ectodomain of the protein which mediates interaction with the target membrane. Biochemical, genetic, and morphological studies on HA and its bromelain fragments suggest that the new hydrophobic principle contains the N-terminal peptide of the HA2 subunit. The ectodomains of the trimer dissociate from each other after acid treatment, but the intact HA remains associated via their C-terminal anchors. Preliminary morphological data, moreover, suggest that oligomeric structures consisting of several HA molecules may be involved in the fusion reaction.

In Semliki Forest virus spike glycoproteins, which are heterotrimers of glycopolypeptides E1, E2 and E3, the acid-induced conformational change appears to be different. As judged by alterations in protease sensitivity, both E1 and E2 undergo irreversible changes in conformation when exposed to a pH below 6.2. In addition to low pH, the change in E1 is dependent on the presence of cholesterol (2). This dependence correlates with the cholesterol requirement observed for fusion of Semliki Forest virus with artificial bilayers (3). No evidence for exposure of hydrophobic peptide moieties, similar to that of Influenza HA, is obtained with the isolated ectodomains of E1 and E2. Thus the two spikes of the two viruses appear to induce fusion by distinctly different mechanisms; one exposes a hydrophobic peptide, the other may expose sterol binding sites.

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Supported by a grant (AI 18599) from NIH and by the Swebelius foundation.

G11 THE MECHANISM OF ENVELOPED VIRUS-CELLULAR MEMBRANE FUSION, F.R. Landsberger, R.A. Demel*, and P.B. Sehgal, The Rockefeller University, New York, NY and *The Rijksuniversiteit Utrecht, the Netherlands.

The entry of enveloped viruses into cells requires membrane fusion which is induced by a virion-associated protein. It appears that virus-induced membrane fusion and lipid transport are closely related. Using lipid monolayer techniques, it was found that Sendai virus as well as influenza virus have lipid transport activity. Egg-grown Sendai virus has its fusion (F) protein in the active form and the virions have infectivity, exhibit membrane fusion activity, and are hemolytic. However, MDBK-grown Sendai virus has most but not all of the F protein in the inactive precursor form. Egg-grown Sendai virus has active phosphatidylcholine specific transport activity. In contrast, the rate of lipid transport catalyzed by MDBK-grown Sendai virus is significantly reduced. While parainfluenza viruses have significant membrane fusion activity at pH of 7.4, the membrane fusion induced by influenza virions, occurs around pH 5.5. Consistent with this, influenza virions mediate phosphatidylcholine transport over a very narrow acid pH range. To determine whether there is a structural similarity between the viral fusion proteins and cellular lipid transport proteins, Seller's TT algorithm was used to find the best sequence alignment between the primary amino acid sequences of these proteins. The alignment between PCTP, a phosphatidyl- choline specific transport protein isolated from beef liver, and the hemagglutinin (HA), which is the fusion protein of influenza A, B, and C, contains a set of glutamic acids which seem to be "preserved" between PCTP and HA but are lacking in the sequences of F. The region of the PCTP molecule found to align with the viral fusion protein has been shown to interact with the lipid fatty acid and forms the long helix along the axis of the HA spike extending out from the viral lipid bilayer. In the monolayer studies, it was also found that the lipid transport by Sendai virus requires calcium. Previously, it was held that Sendai virus induced-membrane fusion does not require calcium. Careful studies recently initiated indicate that Sendai or influenza virus induced hemolysis, which is a sensitive assay for membrane fusion, does require calcium. These data suggest a rather interesting model relating virus fusion with lipid transport. As the pH is lowered, the acidic residues of the influenza HA become discharged and with it the helical region becomes more hydrophobic. For a given lipid in the target membrane there is energetically very little difference between its own lipid bilayer and the hydrophobic region within the viral fusion protein. This model satisfies the criteria for membrane fusion: the dehydration of the interface region of the two membranes and the destabilization of at least one of the membranes. Thus, it is suggested that lipid transport and membrane fusion activity are intimately related activities.

Complement-Mediated Lysis of Cells

G12 ANTI-LYTIC EFFECTS OF POLY-C9 AND CYTOXICITY MEDIATED BY C9-DERIVED PEPTIDES, Alfred F. Esser, Laboratory for Structural Biology, Department of Comparative & Experimental Pathology, University of Florida, Gainesville, FL 32610.

Formation of the "classical complement (C) lesion", which was proposed to be a transmembrane channel effective in target lysis, requires aggregation of ≈15 C9 molecules into polymeric tubular C9 (poly-C9) (1). We have recently demonstrated that thrombin-cleaved C9 ($C9^n$) will not form tubular poly-C9 nor the "classical lesion" on target cells although it retains its full hemolytic potential (2). Likewise, Gram-negative bacteria are killed equally well by C9 and $C9^n$ (3). Since these results demonstrate conclusively that the circular "lesion" formed by poly-C9 is not required for cytotoxicity two questions arose: (i) How does C9 (together with its receptor C5b-8) kill cells? and (ii) What is the function of poly-C9? Recent experiments have indicated that C9 may lyse cells by a "leaky patch" mechanism (4) akin to melittin-mediated hemolysis. We have found that melittin and C form channels with similar effective pore sizes, lyse red cells before the critical hemolytic volume is reached, and most importantly, anti-melittin antibodies inhibit C9-mediated lysis of EAC1-8 and anti-C9 antibodies inhibit melittin hemolysis. That the cytotoxic capacity of the MAC resides in C9 was demonstrated by formation of single-channels across lipid bilayers (BLM) upon addition of the hydrophobic C-terminal domain of C9, C9b. Furthermore, C9b is effective in releasing markers from liposomes and in dissipating the membrane potential of actively respiring bacterial inner membrane vesicles (3). "Osmotic shock treatment" of bacteria in the absence of an assembled C5b-8 receptor complex but in in the presence of C9 results in cell killing, suggesting a bacterial killing mechanism akin to colicin action . Experimental conditions that render bacteria more resistant to C killing result in formation of poly-C9 again demonstrating that this complex is non-lytic and actually inhibits C-mediated cytotoxicity. Such a function may be of vital importance in the protection of autologous cells from "bystander cell lysis" mediated by C. Nucleated cells escape C attack by immediate exocytosis of MAC complexes in the form of membrane vesicles. This protective mechanism is dimished when poly-C9 formation is inhibited, for example, by using $C9^n$. Thus, we propose that poly-C9 is not a cytolytically-active product but rather represents an inactivation product that limits cell damage to the host. (Supported by NSF Grant PCM-82 42116 and NIH Grant RO1-AI 19478).

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G13 POLY C9 AND POLY-PERFORINS: A COMPARISON OF HUMORAL AND CELLULAR EFFECTOR MECHANISMS, Eckhard R. Podack, Department of Microbiology and Immunology, New York Medical College, Valhalla, NY 10595.

Cytolytic lymphocytes effect target cell destruction by damaging the target plasma membrane and the target nucleus resulting in the release of both cytoplasmic markers (eg ^{51}Cr) and DNA fragments. These two distinct cytolytic activities are localized in cytoplasmatic granules of killer lymphocytes released onto target membranes upon killer target conjugation. Isolated granules, in the presence of Ca, are highly cytolytic for nucleated target cells and cause the release of cytoplasmic and nuclear markers. The granule proteins mediating these two activities have been identified. Perforin 1, a 70-75kD cystein rich granule protein, mediates the release of cytoplasmic markers from target cells by Ca-dependent target membrane insertion, polymerization, and pore formation (160 A diameter). Murine Perforin 1 contains a cystein rich domain that, only after reduction, is recognized by an antibody to reduced and alkyated human C9 (see below) indicating sequence homologies between these proteins.

Target DNA destruction may be caused by a lymphotoxin like activity contained in cytolytic granules. Monoclonal antibodies (provided by Genentech) to lymphotoxin partly block DNA release. It is suggested that granule lymphotoxin gains access to the target nucleus via the poly-Perforin pores.

Pore formation in complement is a function of polymerizing C9. Previous studies suggested homologies between C9, C8α-γ, C7 and C6 (Podack, (1984) J. Biol. Chem. 259:8641). In addition it was proposed that C5b-8 may initiate C9 polymerization by virtue of C9 like structures in C5b-8. Using an antiserum produced in rabbits against reduced and alkylated C9, cross reacting determinants could be detected in C9, C8α, C7 and C6 but not in C5 and C8β. These determinants were detected only after reduction and alkylation of the peptide chains suggesting shared sequences. Affinity purification of the anti C9 antibody on reduced and alkylated C7-Sepharose resulted in the production of an antiserum specific for reduced C9, reduced C8α, reduced C7, reduced C6 and reduced Perforin 1. The unreduced proteins were not recognized by this antibody. The antibody reacted with thrombin cleaved C9a and C9b and with C9 dimers and polymers in their unreduced form. The antibody thus detects a common domain in C6, C7, C8α, C9 and Perforin 1 that is available only after reduction of the proteins or after polymerization of C9. It is concluded that the cystein rich C9 domains are also present in C6, C7, C8α and P1, that they are involved in polymerization of C9 by C5b-8, and in the interaction of C6, C7, C8α-γ and C9 in the heteropolymeric tubule of the membrane attack complex. These domains in P1 may mediate the polymerization of perforins in a Ca dependent reaction. Supported by USPHS grants CA39201 AI21999 and by the ACS IM396.

G14 INTERACTION OF THE MEMBRANE ATTACK COMPLEX OF COMPLEMENT WITH NUCLEATED CELL: STUDIES OF THE CELLULAR DEFENSE RESPONSE FOLLOWING SUBLYTIC COMPLEMENT ATTACK. Moon L. Shin, M.D. Department of Pathology, University of Maryland, Baltimore, MD 21201.

Cytolysis by C occurs when activation of C5-C9 produces heteropolymeric transmembrane channels in the plasma membrane (PL), leading to colloid osmotic deregulation. The interaction of C5b-9 with nucleated cells (NC) is of special interest because these cells resist lysis by unique cellular defense processes. This is best shown by the fact that NC are more resistant to C attack than erythrocytes (E), and that metabolic factors may play an important role in protecting NC following limited C attack. As an initial step to understand the action of C against NC we have performed a dose-response curve of NC death with respect to C6 in C6 deficient serum to determine whether the lytic process conforms to a one-hit mechanism as in E. The dose-response curve of cell death at the endpoint with respect to time was sigmoidal in contrast to the monotonic dose-response curve of ^{86}Rb release, measured as an indication of channel formation, which indicate that NC lysis requires multiple channel formation. For NC to survive limited C channels, these cells must be able to counteract the altered osmotic state by either increasing the rate of ion efflux or reducing the rate of influx. However, both in U937 or Erlich's cells were not more sensitive to C attack after treatment with Quabain to inhibit Na^+/K^+ ATPases when tested in lytic dose-response experiments. Alternatively, to test whether this multi-channel requirement is related to the ability of NC to eliminate C5b-9 from the cell surface, the stability of various C complexes in the PM of Ehrlich cells were examined. Cells bearing C5b-7 or C5b-8 with or without C9 were incubated at 37°C for various time intervals before converting the remaining complexes to lytic C5b-9 channels. These complexes were shown to disappear progressively with different rates at 37°C, but not at 0°C. The fate of C5b-8 on the surface of Ehrlich cells, traced with colloidal gold, revealed that these complexes internalized continuously via endocytic vesicles. The differential rates of disappearance of terminal C complexes are of interest because the disappearance rate correlates with the functional pore size of the various C complexes. We have examined whether the disappearance rate may be related to flux of Ca^{2+}, which has been implicated as a regulator of endocytosis. The extracellular Ca^{2+} concentration influenced the disappearance of C complexes, with the largest pore size to the greatest extent by prolonging disappearance as the Ca^{2+} was sufficiently lowered. In addition, these complexes increased the cellular Ca^{2+} concentration in proportion with the pore size and number of the complex. These results suggest that defense responses in NC following C attack are, at least in part, regulated by Ca^{2+}-dependent metabolic processes to promote cell survival.

Immunotoxins: Strategies for Maximizing Activity and Specificity

G15 ENGINEERING OF DIPHTHERIA TOXIN FOR USE IN IMMUNOCONJUGATES. Lawrence Greenfield and Rick Dovey, Department of Microbial Genetics, Cetus Corporation, Emeryville, California, 94608.

We have investigated the use of portions of the diphtheria toxin in immunoconjugates. AN 831 base pair Sau3AI fragment encoding the diphtheria toxin A fragment (DTA), and a 1454 base pair MspI fragment encoding a CRM45-like protein were cloned into pBR322. By removing the diphtheria toxin secretory leader sequence from the diphtheria toxin gene and replacing the native regulatory region with the lambda P_L promoter and gene N ribosomal binding site, we were able to obtain levels of expression of both clones at a level of 5 to 7% of the total cell protein.

The CRM45-like protein (MspRT) consists of amino acids 1 to 198 of the diphtheria toxin (3 amino acids shorter than CRM45) and therefore contains the cysteine bridge between the A and B fragment. We modified this clone (MspCys) to provide an additional cysteine residue to allow conjugation to antibody. In the MspCys derivative, a cysteine residue was added to the carboxyl end of the protein. A third construct (MspSA) contains a sequence of 14 amino acids between the MspRT protein and the carboxyl cysteine to provide flexibility and distance between the toxin and the antibody in conjugates. All three proteins (MspRT, MspCys, and MspSA) were found to have ADP-ribosylation activities similar to native DTA.

The MspSA protein was conjugated to several of our human antibreast monoclonal antibodies by both disulfide and thioether linkages. Both types of conjugates were found to be cytotoxic to a human breast cell line (MCF-7) but not to a control human fibroblast cell line (HS27F).

G16 RECENT IMPROVEMENTS IN CLINICAL EX-VIVO AND EXPERIMENTAL IN VIVO EFFICACY OF A-CHAIN IMMUNOTOXINS. Franz K. Jansen, Pierre Casellas, Hildur E. Blythman, Bernard Bourrié, Jean Marie Derocq, Danielle Dussossoy and Guy Laurent. Immunotoxin Project, Centre de Recherches Clin Midy-Sanofi, rue Prof. J. Blayac, 34082 Montpellier, France.

The T101 anti-T cell immunotoxin (IT) was used in clinical pilot studies for the elimination of mature T cells from allogeneic bone marrow. Although under experimental conditions a 2.5 log cytoreduction was obtained repetitively, cytoreduction under clinical conditions was insufficient. The lacking efficacy proved to be due to slight changes in pH, influencing the potentiation of the T101 IT by NH4Cl, since the free base (NH3), the active form of potentiation, is highly pH dependant. The necessary NH3 concentrations were found to vary for different target cells and different structures of the Ig carrier.
Higher NH3 concentrations were needed by T lymphocytes than by the leukemia cell line CEM and also with whole Ig IT, assembled with the T101 antibody, as compared to its Fab fragments. By using T101 Fab I and compensating for higher NH3 concentrations under clinical conditions, a new clinical pilot study was envisaged for the elimination of T-lymphocytes in allogeneic bone marrow transplantation.

Two strategies were explored to increase in vivo efficacy of A-chain ITs : 1. the inhibition of the rapid clearance of IT from the bloodstream, 2. the transfer of the in vitro potentiation to in vivo conditions with carrier bound monensin. The rapid clearance of IT was not due to the disruption of the disulfide bridge but to the adsorption of IT through the mannose residues of the A-chain in the liver. The rapid clearance of IT from the circulation could be drastically slowed down and becomes similar to the antibody alone by simultaneous injection of mannan, a polymer of mannose which acts as a competitive inhibitor at the receptor. The in vivo potentiation of ITs was achieved by carrier bound monensin, which maintained high levels of active monensin in the blood for a sufficiently long time. Both strategies considerably increased, the in vivo efficacy of a Thy 1.2 IT on T2 mouse leukemia cells leading to longterm survivors after 150 days and may therefore represent some of the key phenomena, which render ITs more effective under in vivo conditions.

G17 SUBCELLULAR COMPARTMENTALIZATION AND POTENCY OF RICIN A CHAIN CYTOTOXINS, Vic Raso, Department of Pathology, Harvard Medical School, Dana-Farber Cancer Institute, Boston, MA 02115

Several approaches have been taken to examine the specific introduction of toxic ricin A chain into cells by various antibody and ligand carriers. Purified A chain was labeled with ^{125}I before disulfide coupling it to cell reactive molecules such as human transferrin, an iron transport protein, and monoclonal antibodies directed against the transferrin receptor or to the common acute lymphoblastic leukemia antigen (CALLA). ^{125}I-A chain conjugates were found to (1) bind to cell membrane receptors or antigens, (2) leave this exposed position on the surface and enter the protected cell interior, and (3) release back out into the media either spontaneously or by ligand induced cycling. Fractionation of the subcellular contents of disrupted cells on percoll density gradients allowed localization of internalized ^{125}I-A chain conjugates within distinct cytoplasmic compartments. In the case of the transferrin-A chain cytotoxin, (Tf-A) these were non-lysosomal, low density, low pH vesicles which induced $^{59}Fe^{+++}$ to uncouple from the carrier portion of Tf-A.

To estimate the extent of intracellular disulfide cleavage or degradation internalized ^{125}I-labeled species were released from detergent lysed cells and analyzed on anti-A chain and anti-carrier affinity columns. For the most part cytotoxins remained intact after passage into cells and little or no free A chain was detected.

Ultrastructural studies using colloidal gold labeled antibodies directed against ricin A chain permitted localization of ligand and antibody delivered A chain within coated pits, coated vesicles as well as other distinct areas inside the cell.

Monensin, a carboxylic ionophore which potentiates the activity of ricin A chain conjugates by accelerating their ability to damage ribosomes, also increased their effectiveness for killing clonogenic target cells by a factor of 10^4-10^5. This enhancement was not due to a rise in vesicle pH since uncoupling of iron from $^{59}Fe^{+++}$ transferrin was not suppressed in monensin treated cells.

Experiments were designed to test whether endocytotic routes are crucial to the biological action of these cytotoxins. The fact that cells could be rescued from the toxic effects of internalized A chain conjugates by ligand induced receptor cycling suggests that these natural pathways play an integral role in allowing toxin access to ribosomes in the cytosol.

G18 ANTI-TUMOUR EFFECTS OF IMMUNOTOXINS, Philip E. Thorpe, David C. Blakey, Edward J. Wawrzynczak and Firenzo Stirpe. Imperial Cancer Research Fund, London, U.K.

A panel of immunotoxins was prepared by linking monoclonal anti-Thy1.1 antibody with the SPDP reagent to the A-chains of ricin or abrin or to three ribosome-inactivating proteins which act in analogous fashion: saporin, bryodin and Momordica charantia inhibitor (MCI).
Cytotoxicity. The immunotoxins were specifically toxic to Thy1.1-expressing AKR-A lymphoma cells in tissue culture. The concentrations of immunotoxin that reduced protein synthesis by 50% ranged between 10^{-11}M for the abrin A immunotoxin and 10^{-9}M for the MCI immunotoxin.
Antitumour activity. Mice bearing peritoneal AKR-A lymphoma cells were given a single intravenous injection of 0.3 n moles of immunotoxin. The extension in median survival time ranged from 6d for the abrin A immunotoxin (corresponding to 99% tumour cell kill) to 24d for the saporin and bryodin immunotoxins (corresponding to 99.999% tumour cell kill).
Linkage. The low antitumour activity of the abrin A immunotoxin could be rectified by changing the crosslinker from SPDP to 2-iminothiolane. This is because about 1% of AKR-A cells survive exposure to the SPDP-linked immunotoxin (both in vitro and in vivo) but are killed by the 2-iminothiolane-linked immunotoxin. It is possible that the resistant cells have elevated levels of an enzyme capable of splitting the amide bond in the SPDP linkage. Both the SPDP and 2-iminothiolane linkages break down slowly in vivo ($t_{\frac{1}{2}}$ = 8 hours) probably because the disulphide bond is unstable. A new coupling agent, SMBT, has been made which avoids this problem.
Reticuloendothelial recognition. Ricin A immunotoxins are cleared in vivo by the reticuloendothelial cells of the liver. These cells have receptors for the oligosaccharide portion of ricin A-chain. Deglycosylation of the A-chain with metaperiodate and cyanoborohydride prevents clearance of the immunotoxin by the reticuloendothelial system.
Pathology. Linkage of saporin and abrin A-chain to immunoglobulin increases their toxicity to mice by 10-20 fold. Both immunotoxins cause extensive hepatic necrosis whereas neither saporin nor abrin A-chain in unconjugated form cause significant liver damage.

Immunotoxins: Clinical Applications

G19 USE OF PSEUDOMONAS TOXIN AND RICIN A TO CONSTRUCT IMMUNOTOXINS DIRECTED AGAINST ADULT T CELL LEUKEMIA AND OVARIAN CANCER. I. Pastan[1], D. FitzGerald[1], M.C. Willingham[1], T. Waldmann[2]. [1]Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; [2]Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892.

Pseudomonas toxin (PE) has been coupled to a variety of monoclonal antibodies and their activity assessed against appropriate cell lines and in some cases compared with ricin A conjugates. A conjugate of PE with an antibody to the IL2 receptor (PE-anti-Tac) kills HUT 102 cells, a cell line transformed by HTLV-1, which bear the IL2 receptor (FitzGerald et al., J. Clin. Invest. 74: 966-971, 1984). Specific cell killing occurs at 5 ng/ml of PE-anti-Tac. Non-specific cell killing occurs at around 1 µg/ml. PE-anti-Tac is currently being evaluated in patients with adult T cell leukemia.

An ovarian cancer model has been developed by Ozols and Hamilton (Hamilton et al., Cancer Res. 44: 5286-5290, 1984) in which a human ovarian cancer cell line (OVCAR-3) grows in the peritoneal cavity of nude mice and produces massive ascities. MABs reacting with this cell line have been conjugated with ricin or PE and tested in this model. An immunotoxin made using an antibody to the tranferrin receptor injected 5 days after tumor implantation increases animal survival from 30-40 days (untreated) to over 100 days (treated). Immunotoxins containing PE appear to be 5-10 fold more active than their RTA counterparts.

G20 THERAPY OF PATIENTS WITH MALIGNANT MELANOMA USING XOMAZYME-MEL®, A MONOCLONAL ANTIMELANOMA ANTIBODY-RICIN A CHAIN IMMUNOTOXIN: RESULTS OF PHASE I TRIAL, Lynn E. Spitler, Michael del Rio, Archie Khentigan, Patrick Scannon, XOMA Corporation, Berkeley, CA 94710

We conducted a Phase I trial of XOMAZYME-MEL®, a murine monoclonal antimelanoma antibody-ricin A chain immunotoxin, in patients with metastatic malignant melanoma under an FDA-approved protocol. Twenty-two patients were evaluated. The dose of immunotoxin administered ranged from 0.01 mg/kg daily for five days to 1 mg/kg daily for four days (total dose: 3.2 mg to 300 mg). Side effects consistent in most patients were a fall in serum albumin with associated fall in serum protein, weight gain, and edema. In addition, patients experienced mild to moderate malaise, myalgia, loss of appetite, and evening fevers. Reactions consistent with allergic reactions were observed in three patients. The observed side effects were related to the dose of immunotoxin administered in mg/kg/day and were generally transient and reversible. Although this was a Phase I study, encouraging clinical results were observed, even after a single course of a low dose of immunotoxin. We conclude that a Phase II study is warranted to continue the evaluation of safety and efficacy of immunotoxin therapy of malignancy.

G21 IMMUNOTOXINS (IT) IN BONE MARROW TRANSPLANTATION (BMT): ERADICATION OF T CELLS/LEUKEMIA CELLS AND POTENTIAL FOR TREATMENT OF RESIDUAL DISEASE, Daniel A. Vallera, Dorothea E. Myers, John H. Kersey, Alexandra H. Filipovich, and Fatih M. Uckun. Dept of Therapeutic Radiology and Laboratory Med./Pathology, U. of Minnesota, Minneapolis, MN 55455.

We have chosen to study intact-ricin IT because of their selectivity, potency, and fast kinetics. We feel they have great potential for elimination of GVHD-causing cells from donor grafts prior to allogeneic BMT and for purging residual leukemic cells from grafts prior to autologous BMT. We have focused on devising better more informative strategies for the biological testing of IT. We have used a clonogenic assay by limiting dilution to determine the selective cytotoxicity of anti-CD5 IT against leukemic T cell lines. We found that intact-ricin IT inhibited 5 logs of clonogenic T leukemia cells, while A chain IT were less effective inhibiting only 1 log of clonogenic cells. Anti-CD5 A chain plus the potentiator monensin elicited 4 logs of kill. The most impressive levels of cytotoxicity were obtained when IT were combined with DNA alkylating reagents. This approach resulted in 6.2 logs of kill without inhibiting human stem cells as measured by in vitro CFU-GEMM assay and 7.4 logs at higher drug concentrations that proved clinically safe. We have also determined the effect of mixtures of IT on cell lines. Mixtures of IT did not enhance IT kill as compared to individual IT in assays using homogenous cell lines, but did in assays of T cell function involving heterogenous cell populations. We are also committed to determining which IT are the best for treatment based on the reactivity of IT with leukemic progenitor cells measured in novel colony assays. These assays are important since data obtained using cell lines may not accurately reflect the efficacy of IT against clonogenic blasts from ALL patients. We found that T-lineage blast progenitor cells express the CD2, CD5, and CD7 surface antigens and are sensitive to ricin. Thus, IT directed against these determinants show potential for clinical use in autologous BMT for T-ALL.

Our findings have resulted in a phase I trial using IT to purge leukemic cells. Seven patients received IT treated autotransplants since March of 1984 (5 T-ALL and 2-T lymphoma). This early study shows that IT represent a safe, specific, and simple method of purging bone marrow of residual leukemic cells. We have also undertaken a phase I evaluation of IT to eliminate T cells from donor marrow in matched sibling transplants for GVHD prophylaxis. A mixture of 3 anti-T cell intact ricin IT were used to purge allogeneic bone marrow. Our studies show that IT purging presents little risk of toxicity and results in shortened hospital stays compared with conventional prophylactic regimens. Most importantly, of 17 patients, mostly adults, there was no occurence of severe (Grade III, IV) GVHD. Graft failure was a problem in 4 of the patients. This has been similarly observed in centers employing other T cell depletion strategies. We are currently evaluating IT synthesized using hemitoxins. These reagents may have therapeutic potential in the systemic treatment of leukemia patients.

Monoclonal Antibodies: Clinical Applications

G22 HUMAN MELANOMA ANTIGENS AS TARGETS FOR IMMUNOTHERAPY, R. A. Reisfeld, D. A. Cheresh, C. J. Honsik, and Y. Koyama, Scripps Clinic and Research Foundation, 10666 No. Torrey Pines Road, La Jolla, CA 92037.

The major gangliosides expressed on surfaces of the human melanoma cells, GD_2 and GD_3, are involved in tumor cell-substratum interactions, as indicated by two findings. First, these antigens were found heavily expressed in adhesion plaques examined by indirect immunofluorescence; second, monoclonal antibodies directed to GD_2 and GD_3 inhibited attachment of melanoma cells to fibronectin. Preliminary results suggest that the gangliosides interact with a specific protein receptor for fibronectin. Amounts of GD_3 specifically increased on metastatic versus primary melanoma cells derived from the same patients, as determined by fluorescence-activated cell sorter analysis. Moreover, human melanoma cells that are selected for their ability to establish pulmonary metastases in athymic (nu/nu) mice also express an increased amount of GD_3 on their surfaces. Intrinsic radiolabeling of such cells indicates that this increased expression is caused by an increase in the biosynthetic rate of GD_3 in metastatic versus primary melanoma cells.

GD_3 is a relevant target antigen for monoclonal antibody-mediated cancer therapy, since anti-GD_3 monoclonal antibodies MB 3.6 and 11C64, can effectively lyse melanoma tumor cells in the plasma of human complement and by antibody-dependent cellular cytotoxicity (ADCC). Effector cells isolated from the peripheral blood of either normal individuals or cancer patients were equally suitable for ADCC. Cells with natural killer (NK) activity apparently constitute a cell population that affects ADCC, since only large granular lymphocytes, i.e. NK cells, are reactive; monocytes and enriched T cell populations are inactive.

Monoclonal antibodies MB 3.6 and 11C64 not only affect growth of human melanoma cells in vitro but also effectively suppress the growth of such cells in vivo. Specifically, anti-GD_3 antibodies per se inhibit the establishment of tumors in nude mice whereas a single intravenous injection of antibody (400 ug) together with 2 X 10^7 splenocytes from BALB/C mice completely destroys established (~40mm^3) human melanoma tumors in such animals. Together, these data indicate that ganglioside GD_3 represents a most suitable target for melanoma tumor killing in a nude mouse model system and also suggest that the antibody/effector cell approach may eventually provide a new modality for the clinical treatment of human malignant melanoma.

G23 THE PHARMACOLOGY OF MONOCLONAL ANTIBODIES, John N. Weinstein, Christopher D.V. Black, Robert J. Parker, O. Dile Holton, III, Renee Eger, Jacques Barbet, Andrew M. Keenan, Susan M. Sieber, and David G. Covell, National Cancer Institute, NIH, Bethesda, Md 20892.

High specificity is useful in vivo only if an administered antibody or antibody conjugate reaches the intended target and interacts with it effectively. Pharmacologic factors (at the cellular, whole organ, and whole body levels) are of paramount importance. Some of those factors can be analyzed in a straightforward, qualitative way; others are more subtle and require formal, quantitative analysis of the physiologic systems involved (1,2). To aid in that analysis, we have used the SAAM computer programs to construct physiologically-based models with which to assess our own (and others') data from animal and clinical experiments. The models have been applied to data obtained after intravenous and subcutaneous antibody administration. Both routes will be discussed.

Models were constructed in the context of experiments on the pharmacodistribution of IgG, $F(ab')_2$, and Fab' after i.v. and s.c. injection in mice. The bindable antibody was a murine IgG_1 directed against the allelic B-cell determinant LyB8.2. MOPC 21 and its fragments served as isotype-matched controls and yielded reference metabolic data on non-bindable Ig. The results shed light on the complex trade-offs between the distribution, clearance, and binding properties of the different fragments (for use in design of in vivo applications). As expected, all of the salient processes were faster for Fab' fragments than for the larger moieties, but that will not always lead to favorable results. As in our past studies with other antigenic targets on normal cell types (3,4), s.c. administration was much more efficient than i.v. injection for delivery of antibody to regional lymph nodes.

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G24 HUMAN TUMOR NECROSIS FACTOR AND LYMPHOTOXIN: THEIR STRUCTURAL AND FUNCTIONAL SIMILARITIES, Bharat B. Aggarwal and Thomas E. Eessalu, Department of Protein Biochemistry, Genentech Incorporated, 460 Point San Bruno Blvd., South San Francisco, CA 94080

Tumor Necrosis Factor (TNF) is a cytotoxic activity, known to be secreted into the sera of animals injected sequentially with bacillus Calmette-Guerin and endotoxin.[1] The activation of lymphocytes causes the production of another cytotoxic activity called lymphotoxin (LT).[2] We have used a human monocytic cell line HL-60 and a human lymphoblastoid cell line RPMI-1788 as a source of proteins that we have designated to be TNF and LT respectively. Their relationship to the protein(s) previously reported to be responsible for cytotoxic activity is ambiguous. Both TNF and LT have been purified to homogeneity from these cell lines and their complete amino acid sequence determined. TNF is a non-glycoprotein containing 157 amino acid residues whereas LT is a glycoprotein and consists of 171 amino acids. The carbohydrate portion of the LT molecule appears not to be required for its biological activity. The amino acid sequence of TNF is 36 percent identical and 51 percent homologous to LT. Secondary structure predictions based upon amino acid sequence indicated that both proteins contain greater than 60 percent β-sheet and less than 20 percent α-helix. The hydropathy profiles of LT and TNF show that they exhibit hydrophilic amino terminal and hydrophobic carboxyl terminal ends. Using amino acid sequence information on LT and TNF, both cytokines have been cloned and expressed by recombinant DNA methods.

Both TNF and LT are known to inhibit the growth of various tumor cells in vitro and in vivo. The antitumor activities of these cytokines are synergistically potentiated by interferons. Our recent results indicate that TNF and LT interact with cells through a specific, single class of high affinity receptors ($K_d \geq 10^{-10}$M) and there are on the average approximately 2000 receptor sites per cell. Furthermore, the number of receptors can be induced 2-3 fold by interferon without any change in their affinity constant. The binding of TNF to cells can be displaced quantitatively by LT, suggesting that these two molecules share a common receptor. Thus both structural and functional characteristics indicate that LT and TNF are closely related molecules released by two different cell types of the immune system under differential control. Due to these similarities, we have renamed LT as TNF-β.

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INVITED TALK AT THE "1986 UCLA SYMPOSIUM ON MEMBRANE MEDIATED CYTOTOXICITY"

Cytotoxic Effector Molecules of Leukocytes

G25 ROLE OF SOLUBLE CYTOTOXIC FACTORS IN NK CELL MEDIATED CYTOTOXICITY, Benjamin Bonavida, Susan C. Wright, Robert Roozemond*, Stan Wilbur, and Scott Graves, Dept. Microbiology and Immunology, UCLA School of Medicine, and *The University of Amsterdam, The Netherlands.

Studies derived from our laboratory have provided evidence for a role of NKCF in the mechanism of NK CMC reaction. Rodents and human NK effector cells cocultured with NK-sensitive tumor cells release NKCF into the supernatant. These factors are selectively cytotoxic for NK-sensitive target cells and also species-specific. Several lines of evidence have established a close correlation between the known characteristics of NK cells and the properties of NKCF. Based on these studies, we have proposed a model consisting of various steps and stages which account for the NK lytic mechanism. Initially the NK cell recognizes and binds the target cell, the target cell delivers a signal to the effector cells to activate the NKCF release mechanism. Once the NKCF are released, they bind the target cells via NKCF binding sites. Subsequent to binding of NKCF, the target cells are lysed in the lymphocyte-independent phase of the cytolytic reaction. Experimental evidence in support of the model as well as characteristics of the various stages involved in the NK lytic pathway will be presented. Also, the relationship of NKCF to other lytic mediators will be discussed.

G26 LEUKOREGULIN, LYMPHOTOXIN AND INTERFERON TRANSMEMBRANE SIGNALS AND REGULATION OF CELL GROWTH, Charles H. Evans, National Cancer Institute, Bethesda, MD 20892.

The role of plasma membrane ion fluxes in the ability of lymphokines to modulate target cell membrane stability and cell proliferation was examined by flow cytometic analysis of cell surface perturbations indicative of membrane destabilization. pI 5.1 leukoregulin, pI 7.0 lymphotoxin, and interferon were isolated by ion exchange, isoelectric focusing and molecular sizing chromatography from lymphokines produced by phytohemagglutinin stimulated normal human lymphocytes. Increased membrane permeability commencing within five minutes of lymphokine exposure of K562 human erythroleukemia cells was quantitated by measuring the efflux of intracellular fluorescein or influx of propidium iodide using 488 nm argon laser excitation in a FACS IV cell sorting flow cytometer or using mercury arc excitation in a FACS Analyzer. Membrane permeability increased in proportion to leukoregulin concentration but remained unperturbed in the presence of either lymphotoxin or interferon. By two hours 0.25-30 units of leukoregulin/ml induced a 10-90 percent change in permeability. Similar changes in K562 plasma membrane permeability were effected by Ca^{++} ionophores A23187 and X-537A but not by the Na^{+} ionophore monensin nor the K^{+} ionophore valinomycin. Ca^{++} ionophores and intracellular Ca^{++} mobilizers oaubain and amphotericin B, moreover, enhanced whereas calmodulin inhibited leukoregulin action. Neither the Ca^{++} channel blockers nifedipine or verapamil nor the Na^{+} or K^{+} transport blockers amiloride and atracyloside altered membrane permeability or enhanced leukoregulin activity. Further evidence for increased Ca^{++} flux during leukoregulin membrane destabilization was an increase in intracellular free Ca^{++} measured by the Ca^{++} sensitive fluorescent probe quin2. Kinetic analysis of target cell volume, forward and right angle light scatter, fluorescein efflux and propidium iodide influx, moreover, revealed that the pattern induced by leukoregulin is unique. Within minutes of target cell interaction leukoregulin initiates a series of membrane perturbations characterized by increased Ca^{++} flux, membrane permeability, and altered cell surface conformation. One unit of leukoregulin produces a 50 percent reduction in the proliferation of 10^4 K562 cells and affects each phase of the cell cycle. As many as 5×10^4 I.U. of γ-interferon and 10^2 αL929 cytolytic lymphotoxin units fail to alter K562 proliferation or membrane permeability. Leukoregulin is produced by natural killer cells and by itself increases the sensitivity of target cells to natural killer cell cytolysis. These activities and leukoregulin's high specificity for tumor cells indicate its potential as one of the early mediators effecting the destabilization of the target cell membrane in natural cytotoxicity reactions. Increased transmembrane Ca^{++} flux and plasma membrane permeability may be critical initial steps in the ability of leukoregulin to prevent the development of carcinogenesis as well as inhibit the continued proliferation of tumor cells.

G27 LYMPHOTOXINS: ANTI-TUMOR AGENTS AND INDUCERS OF INFLAMMATION. G. Granger, S. Orr, B. Averbook, I. Masunaka and R. Yamamoto. U. of Cal. Irvine, Irvine, CA.

Human B cells, NK cells, and specific and nonspepcific cytolytic T cells can release a lymphotoxin(LT) termed alpha. Human alpha LT is a protein that appears in two molecular forms. The present studies were performed with a purified, dimeric alpha LT composed of one 72,000 Molecular Weight(MW) heavy(H) chain and a 25-27,000MW light(L) chain. Immunologic and structural studies indicate the L and H chains are distinct peptides. This material causes dose dependent growth inhibition or necrosis of Meth A tumors growing in Balb/c mice when administered systemically or into the tumor. Treated animals remain tumor free for one year and are resistant to rechallenge with Meth A cells but are succeptable to NS-1 tumors. The molecule has a 2-3 hr half life in normal and tumor bearing Balb/c mice. Parenterally administered LT does not selectivly localize in tumor tissue and is rapidly sequestered in and excreted by the kidney. Histologic studies of injected tumors shows an early influx of host PMN and later of mononuclear cells. This LT form induces a strong cellular infiltration when injected into the skin of normal mice and rabbits. Thus material has anti-tumor effects and appears to be an inducer of inflammation.

Cell-Mediated Killing and Clinical Applications

G28 CYTOTOXIC T CELL-ASSOCIATED TRANSCRIPTS, Pierre Golstein, Jean-François Brunet, Magali Roux-Dosseto, William R. Clark, Tariq M. Haqqi, Anne-Marie Schmitt-Verhulst and Marie-Françoise Luciani, Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille Cedex 9, France

Molecular biology techniques could be used in two ways when studying the mechanism of T cell-mediated cytotoxicity. A first way is to clone genes coding for known cytolytic molecules (such as lymphotoxins, cytolysin/perforin). A second way is to try to detect molecules, not necessarily known, which might be involved in cytotoxicity, on the basis of differential transcription of the corresponding genes in cytotoxic T cells and not in other cells.

Following the latter approach, we made a differential (Tc-B) cDNA library and subjected the recombinant clones to three successive rounds of screening with a range of radioactive cDNA probes derived from various cytotoxic and non-cytotoxic cell populations. Ultimately selected recombinant cDNA clones were grouped into 3 CTLA families according to cross-hybridization pattern and transcript size. Additional checks for cytotoxic T cell association were made in Northern blot experiments. These results will be presented, as well as those of preliminary studies on structure and expression-function relationship.

G29 ROLE OF NATURAL KILLER CELLS IN PREVENTION AND THERAPY OF METASTASES, Ronald B. Herberman, Pittsburgh Cancer Institute, Pittsburgh, PA 15213.

There is increasing evidence that natural killer (NK) cells play an important role in the prevention and therapy of tumor metastases. Selective deficits in NK activity have been associated with a substantial increase in the number of metastases in organs usually susceptible to metastases by a particular tumor, and also with the development of metastases in organs usually resistant to formation of metastases. Conversely, restoration of deficient NK activity or augmentation of NK activity above baseline levels results in increased resistance to development of metastases. These levels of NK activity in the major organs which are susceptible to metastases are more relevant than the levels of NK activity in the peripheral blood or spleen. It has recently been shown that administration of various immunomodulators, including interferon and interleukin 2, can substantially augment organ-associated NK activity. Such information should lead to new strategies for prevention and therapy of metastases. In such therapeutic approaches, the main consideration is the utilization of a dose and schedule of the immunomodulator which will induce repeated augmentation or sustained high levels of NK activity. An alternative strategy for utilization of NK cells for therapy is to stimulate these effector cells in vitro and then adoptively transfer them to the tumor bearing-individual. The majority of IL 2-stimulated cytotoxic effector cells, as well as the interferon-stimulated effector cells, have been shown to have the characteristics of activated NK cells. When such cells are transferred to tumor-bearing individuals, combined with repeated administration of IL-2 to maintain cytotoxic effector function in vivo, substantial therapeutic results have been noted in various tumor models. Therapy has been most effective when tumor-burden is first reduced by appropriate cytoreductive therapy. Presumably because of limitations in the circulation pattern of the transferred cells, administration of effector cells to each of the tissue compartments affected by tumor has been necessary to induce cures.

G30 IMMUNOTHERAPY OF CANCER USING LYMPHOKINE ACTIVATED KILLER (LAK) CELLS AND INTERLEUKIN-2 (IL 2)[1] Steven A. Rosenberg and Stephen E. Ettinghausen, Surgery Branch, National Cancer Institute, Bethesda, MD 20892

The incubation of murine splenocytes or human lymphocytes in IL-2, a lymphokine produced by T helper cells, generates LAK cells which are capable of lysing a wide variety of fresh, natural killer (NK) resistant tumor cells but not normal cells in short term chromium release assays. LAK cells are distinct from NK cells and CTL. We have developed models of pulmonary and hepatic metastases in mice and, with these models, have established the anti-tumor efficacy of LAK cells against immunogenic and non-immunogenic sarcomas, melanomas and carcinomas. A single tumor cell suspension is injected intravenously (iv) to generate lung metastases or intrasplenically via a left subcostal incision to create liver metastases. On day 3 after injection, when established metastases are evident histologically, mice receive 10^8 LAK cells iv followed by a second LAK cell dose on day 6. From day 3-8, 5000-100,000 units of IL-2 in Hank's saline (HBSS) are given intraperitoneally (ip) 3 times a day (tid). On day 14, mice are ear-tagged and randomized. The lungs or livers are harvested and counted blindly before the code is broken and data analyzed. In 27 experiments with 3 day pulmonary sarcoma metastases, the combination of LAK cells plus 20,000-50,000 units of IL-2 produced an 88.3±1.9% reduction of metastases when compared to HBSS alone. Similar results were obtained in the liver model. No significant reduction of metastases was observed when either LAK cells or IL-2 at these doses were used alone or fresh or cultured (without IL-2) splenocytes were substituted for LAK cells. Maximal in vivo activity of LAK cells was generated after 3 days of incubation in IL-2. Allogeneic LAK cells were as effective as syngeneic cells in vitro and in vivo in the lung model. Titrations of both IL-2 dose and numbers of LAK cell infusions and LAK cells/infusion showed a direct relationship with anti-tumor activity in both models. Therapy with LAK cells plus IL-2 yielded a significant prolongation in survival as compared to controls.

Since IL-2 was required for successful LAK cell therapy, we studied the ability of IL-2 to stimulate tissue expansion of donor LAK cells in tissues. In vivo proliferation was assessed by the ip injection of ^{125}I-iodo-deoxyuridine (IUdR), a thymidine analog incorporated into the DNA of dividing cells. In response to injected IL-2, significant expansion of donor LAK cells occurred in the lungs, liver, kidneys, spleen and mesenteric lymph nodes of mice preirradiated to eliminate proliferation by endogenous lymphocytes. LAK cells recovered from lungs of mice receiving LAK cells plus IL-2 showed at least a 32-fold increase in number of lytic units per lungs over those of control animals. Because of the consistent success of this treatment with a broad range of tumors in the murine system, a trial of LAK cells plus IL-2 in the therapy of humans with cancer is presently underway.

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Liposomal Delivery Systems and Applications

G31 IMMUNOLOGIC AND METABOLIC CONSEQUENCES OF LIPOSOME-CELL INTERACTIONS, Carl R. Alving, Nabila M. Wassef, Marti Jett, and William E. Fogler. Department of Membrane Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307-5100

The influence of phospholipid metabolism on cell functions has been probed with liposomes. Numerous systems have been described in which external stimuli, such as receptor-agonist interaction, induce increased turnover of intracellular phosphatidylinositol (PI). We have devised methods, using liposomes containing PI or other phospholipids, to suppress increased PI turnover. For example, complement-dependent phagocytosis of liposomes by macrophages is associated with increased PI turnover, but phagocytosis and increased PI turnover both were suppressed when the liposomes contained PI. We now describe three systems in which phagocytosis and PI turnover are dissociated. In each case enhanced phagocytosis occurred as expected, but increased PI turnover was suppressed. We conclude that the "messenger" function that is often associated with increased PI turnover can be induced by opsonized liposomes. However, PI turnover associated with phagocytosis also can be readily suppressed by appropriate liposomes without adversely influencing phagocytosis. It appears that the mechanism responsible for PI turnover is an independent unit that can be modulated by extracellular stimuli.

We have also examined the rate of turnover of phospholipids in murine peritoneal macrophages during the attachment and spreading phase after initiation of culture. Certain phospholipids (PC, PE, PS) undergo an increase in ^{32}P labeling while other phospholipids (PA, PI, PIP, PIP_2) do not show a parallel increased incorporation of radiolabel. It is likely that PC, PE, and PS represent "building block" phospholipids that are synthesized rapidly during periods of increased plasma membrane synthesis. This observation strengthens the concept that "messenger" and "building block" systems of phospholipids can exist independently in the cell membranes, and each may be independently sensitive to extracellular stimuli such as opsonization and phagocytosis.

The effects of liposomes on nonphagocytic cells were also investigated. Liposomes containing plant PI (but not those with animal PI) are cytotoxic to 15 tumor cell lines, but are not cytotoxic to 4 normal cells. We now know, that "cytotoxic" phospholipids (PI or PC containing sn-2 linoleic or arachidonic acid) are taken up by tumor cells to a much greater extent than "noncytotoxic" phospholipids (PI or PC containing sn-2 oleic acid). Degradation

of greatly increased amounts of PI or PC by phospholipase A_2 produces high levels of free fatty acid and lysophospholipid, both of which compounds exert considerable cytotoxicity.

We conclude that liposomes can gain access to and influence at least three independent cellular "compartments" that are closely linked to phospholipid turnover and metabolism. These compartments can be characterized as representing "messenger", "building block", and "metabolic" functions of cells and cell membranes.

G32 LIPOSOMAL DELIVERY OF A WATER-SOLUBLE DRUG COVALENTLY ATTACHED TO LIPID BILAYERS. Stephen C. Kinsky and Keiichiro Hashimoto, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206.

Liposomes, containing methotrexate (MTX) trapped in the aqueous compartments, have been extensively investigated as models for vesicle-mediated drug delivery. These studies have revealed several obstacles associated with potential liposomal therapy, including the problem of drug leakage. In the case of methotrexate, the latter can be circumvented by incorporating the drug into lipid bilayers as MTX-γ-DMPE (an amphipathic derivative in which MTX is linked via its γ glutamyl carboxyl group to the amino function of dimyristoylphosphatidylethanolamine (DMPE)) (1). Liposomes, prepared with MTX-γ-DMPE, were 10-100 fold more effective than liposomes, made with either MTX-α-DMPE or MTX-α, γ-diDMPE, in inhibiting the proliferation of both mouse and human cell lines (as measured by the incorporation of tritiated deoxyuridine into DNA) (2). Most importantly, MTX-γ-DMPE sensitized liposomes do not employ the ubiquitous methotrexate transport system to enter cells in spite of the fact that MTX residues, which are anchored to liposomal bilayers in this manner, are accessible to other proteins that have binding sites for the drug (e.g., the target enzyme, dihydrofolate reductase (DHFR), and anti-methotrexate antibodies) (2). Thus, these liposomes are able to block growth of cells that are resistant to methotrexate because of a defective transport system (3). Available evidence also suggests that the liposomes may partially reverse methotrexate resistance due to overproduction of DHFR, perhaps as a consequence of the intracellular accumulation of phosphorylated metabolites of MTX-γ-DMPE. Current studies focus on the ability of antibodies to target the liposomes to specific cells, and particularly the influence of antibody direction on the mechanism of liposomal uptake (endocytosis vs. fusion) and subsequent metabolism of MTX-γ-DMPE. These experiments utilize several new iodoacetylated and biotinylated phospholipid derivatives (to be described) for the preparation and isolation of liposomes with attached monoclonal antibodies.

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G33 ANTIBODY-DIRECTED LIPOSOMES FOR TARGETING OF DRUGS TO SPECIFIC CELLS D. Papahadjopoulos, T. Heath, R. Debs, K. Matthay, and R. Straubinger.
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Drug delivery to cancer cells with antibody-directed liposomes is a promising method to increase specificity and efficacy of cancer therapy. Toward this end, we have developed methodology for conjugating immunoglobulins on liposomes (1) and have studied the conditions necessary for optimal delivery of cytotoxic drugs into the recipient target cells (2).

We have described the following important parameters for enhancing the efficacy of antibody-directed liposomes in drug delivery: i) Liposome-dependent cytotoxic agents: Drugs such as methotrexate-γ-aspartate (3) and 5-fluoro-orotic acid (4), which are unable to enter cells without a carrier, thus diminishing the non-specific effects of the drug itself. ii) Multivalency of liposome interactions: Antibody-conjugated liposomes have a higher valency than the soluble antibody and bind to cells with up to 1,000-fold higher affinity constant (5). iii) Recognition of multiple ligands: Liposomes which interact with more than one type of ligands on the cell surface show marked advantages in both cell association and cytotoxicity (6). iv) Optimal liposome size for drug delivery: Optimal diameter ranges from 0.05 to 0.1 micron, depending on target cell type (7).

We have used antibody-conjugated liposomes to increase localization at specific target tissues present within the vascular system. Liposomes conjugated to MRCOX7 (anti-Thy 1.1) monoclonal antibody showed enhanced uptake by the lymph nodes of AKR-J (Thy 1.1) mice compared to AKR-Cu (Thy 1.2) mice. This was as expected since Thy 1.1 is an antigen expressed on T lymphocytes present in blood, thymus, lymph nodes and spleen of Thy 1.1 positive (AKR-J) mice. We have also found, however, that the presence of liposome-bound antibody increases liposome clearance by the liver, while decreasing liposome levels in a number of other organs.

It appears from the above results that direct accessibility to the target cells, and by-passing or taking advantage of the hepatic clearance should be part of any strategy for in vivo liposome targeting. On the basis of the above conclusions, we have chosen ovarian carcinoma and liver metastasis as the most promising systems for future therapeutic use of antibody-targeted liposomes.

References.
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Genetics of Cell Recognition and Killing

G34 A MOLECULAR ANALYSIS OF THE CYTOLYTIC T LYMPHOCYTE RESPONSE, Steven J. Burakoff, Division of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

Cytolytic T lymphocyte (CTL)-mediated lysis of an appropriate target cell is a complex cell-cell interaction with multiple functional requirements. Effective lysis of the target cell requires CTL adhesion to the target cell, antigen recognition, the triggering of intracellular activation and the discharge of the lytic mechanism. On a molecular level, several T cell surface structures have been associated with these functions. CD4/CD8, CD2, CD3, LFA-1 and LFA-3 are membrane molecules that do not directly bind antigen, but may facilitate recognition and lysis of the target cell. To investigate these function-associated molecules, we have used a variety of genetic and monoclonal antibody (MAb) techniques. MAb to all six molecules inhibit CTL-mediated cytolysis in the absence of complement. In addition to inhibiting cytolysis, MAb to CD3 have been shown to trigger nonspecific CTL-mediated killing. The ability of these MAb to influence CTL function, whereas MAb to most cell surface molecules do not, suggests that these molecules participate in CTL function. A complementary approach to MAb inhibition has been the use of genetic variants. Spontaneous phenotypic variants obtained from both murine T cell hybridomas and human CTL clones have helped clarify the function of the CD4/CD8 molecules. The heritable LFA-1 deficiency syndrome has permitted detailed study of the LFA-1 molecule on human CTL clones. In the absence of genetic variation, a somatic cell hybrid approach has been used to evaluate the participation of LFA-3 in the CTL-target cell interaction. By using MAb, genetic variants, and DNA-mediated gene transfer, we have been able to clarify the function of these cell surface molecules and further define their role in the CTL-target cell interaction.

G35 INVOLVEMENT OF PHOSPHOLIPASE A_2 IN THE MECHANISMS OF CELL ACTIVATION AND KILLING, Fusao Hirata, Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, Maryland 20892

The cytolytic reaction mediated by cytotoxic T lymphocytes (CTL) and natural killer (NK) cells involves a sequence of binding to target cells, postbinding cytolytic events and recycling of effector cells. Quinacrine, p-bromophenacyl bromide, tetracaine, and naturally occurring phospholipase inhibitory protein (lipocortin) inhibit dose-dependently this cytolytic reaction as well as phospholipase A_2 activity in vitro. The inhibition of this reaction required the presence of these compounds during the early period of the assay and the treatment of the effector cells but not of the target cells exhibited greater inhibition. These agents did not block the binding of the effector cells to the target cells. Since all these compounds inhibit phospholipases by different mechanisms, these results suggest that phospholipase(s) is involved in the cytolytic reaction. In addition to inhibitory effects on the acute phase of the reaction, these phospholipase inhibitors suppressed the generation of CTL stimulated by allogenic non-T cells, but they failed to reduce the secondary prolifirative response of CTL to the same stimulation. These results suggest that phospholipase inhibitors do not block the recognition of antigens by lymphocytes, while they inhibit the mitogenic response of T cells. Taken together, we assumed that phospholipase(s) is involved in the postbinding events of cellular activation.

Cytotoxic Proteins: Structure and Activity Receptors; Receptor-Mediated Entry Mechanisms; Membrane Interactions of Toxins and Viruses Structure; Function of the Complement System

G36 A NOVEL RICIN ISOTOXIN OF LOW TOXICITY, Will Bloch, Ross Cox, Greg Groetsema, Charles Vitt, Walter Laird, Robert Ferris, Margaret Moreland, and David Birch, Cetus Corporation, Emeryville, CA 94608.

Affinity chromatography on any of three very different affinity adsorbents has permitted fractionation of the alkaline isotoxin, ricin E, into two forms which we call E1 and E2. Ricin E1 has the structural and functional properties previously assigned to ricin E. It has three charge types with pI values of 8.7, 8.6, and 8.2, mouse toxicity similar to that of ricin D, cytotoxicity between 1 and 100% of that of ricin D (depending on cell line), and a very low affinity for agarose. Ricin E2, the less abundant form, has 2-5% of the cytotoxicity and 1% of the mouse toxicity of ricin E1 and has significantly higher affinity than ricin E1 for agarose (lower than that of ricin D under some elution conditions). The modest structural difference between ricins E1 and E2 appears to reside entirely in the B chains, which have different apparent MW values but identical isoelectric focusing patterns and carbohydrate compositions. Anti-human-breast-tumor immunotoxins containing ricin E2 show encouragingly high specific cytotoxicity and low mouse toxicity. They perform better in these regards than corresponding immunotoxins containing ricin E1.

G37 FORMATION OF INFLUENZA VIRUS PARTICLES LACKING HEMAGGLUTININ ON THE VIRAL ENVELOPE: ANALYSIS OF TEMPERATURE-SENSITIVE MUTANTS

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Using temperature-sensitive mutants (ts134 and ts61S) of A/WSN/33 strain of influenza virus possessing a defect in the intracellular transport of hemagglutinin (HA), we show that the transport of HA in these mutant virus-infected cells at the nonpermissive temperature (39.5°C) is blocked at the rough endoplasmic reticulum. We demonstrate that the defect is due to a single amino acid substitution in these mutants. We also report that noninfectious virus particles are produced in the mutant virus-infected cells at 39.5°C. These particles are shown to contain all eight viral RNA segments as well as all the structural polypeptides of influenza virus except HA. Compared to the wild type virus made either at 33°C or 39.5° or ts mutants at 33°C, these particles are lighter in density, more fragile and pleiomorphic, and completely lack spikes on the envelope. In the light of these observations we suggest that the presence of HA is not an obligatory requirement for the assembly of the viral components and the budding of influenza virus particles from infected cells. Implications of these results and the possible role of other viral proteins in influenza virus morphogenesis are discussed.

G38 GALα1→4GAL-CONTAINING GLYCOLIPIDS AS MEMBRANE RECEPTORS FOR SHIGA TOXIN AND SHIGA-LIKE TOXIN(S), J. Edward Brown*, Alf A. Lindberg§, Marie Westling§, Karl-Anders Karlsson† and Nicklas Strömberg†, *Walter Reed Army Inst. of Res., Washington, DC, §Karolinska Inst., Stockholm, Sweden and †Univ. of Göteborg, Göteborg, Sweden.

We have examined the glycolipid affinity, cell surface binding and cytotoxicity-associated binding of Shiga toxin from Shigella dysenteriae 1 and of Shiga-like toxin(s) from Escherichia coli. Of various glycolipids tested, galabiosylceramide, globotriaosylceramide, and blood group antigen P_1 bound purified Shiga toxin with high affinity. Synthetic glycoconjugates, containing a terminal galα1→4gal disaccharide linked to bovine serum albumin (gal-gal-BSA), prevented binding of ^{125}I-Shiga toxin to HeLa and vero cell monolayers, whereas galα1→4gal-containing oligosaccharides did not. Gal-gal-BSA also protected HeLa cells from cytotoxic activity when monolayers were incubated at 0°C with mixtures of toxin and gal-gal-BSA for 1 hr, followed by neutralization of unbound toxin and overnight incubation at 37°C. Blocking of binding and cytotoxicity were obtained at near equimolar ratios of gal-gal-BSA with toxin. In addition, the glycoconjugate protected HeLa cell monolayers from cytotoxicity of Shiga-like toxin(s) in sonicates of E. coli of clinical origin, i.e. strain 933 (hemorrhagic colitis), strain H19 (infant diarrhea), strain S/22/1 (infant diarrhea), and strain CL 40 (hemolytic uremic syndrome). Gal-gal-BSA did not alter inhibition of cell-free protein synthesis, a property of the toxin's A subunit. These data suggest that the toxins bind through a multivalent interaction of toxin B subunits with glycolipids in the cell membrane containing a terminal galα1→4gal disaccharide.

G39 STRUCTURE & FUNCTION OF THE YEAST KILLER TOXIN PRECURSOR, H. Bussey, C. Boone, Z. Hong, D. Greene, Biology Department, McGill University, T. Vernet and D.Y. Thomas, Biotechnology Research Institute, National Research Council of Canada, Montreal, Quebec, Canada. H3A 1B1.

The K1 killer toxin of the yeast, Saccharomyces cerevisiae, acts, following cell wall receptor binding, to cause ion-channel formation in the plasma-membrane, and subsequent disruption of the electrochemical potential of sensitive cells. We have recently cloned and expressed in yeast, a cDNA copy of the preprotoxin gene, normally coded on a double-stranded RNA genome. Using site-specific mutagenesis, we have begun an analysis of the functional domains of the precursor, which contains a toxin immunity activity as well as the toxin. The preprotoxin of size 42K, consists of a leader peptide, followed by the alpha and beta toxin subunits, separated by a central 21K gamma glycoprotein. We have identified a hydrophobic, membrane-spanning domain in alpha that is involved in both toxin and immunity activity. This finding suggests that immunity is a property of the toxin precursor, which acts as a competitive inhibitor of mature secreted toxin in killer cells, perhaps by occupying a membrane receptor normally recognised by the alpha region of the toxin, and necessary for toxin action.

G40 MOLECULAR CLONING AND EXPRESSION OF RECOMBINANT RICIN B CHAIN, Ming-Shi Chang, David W. Russell, Jonathan W. Uhr, and Ellen S. Vitetta, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

Ricin A chain-containing immunotoxins (A-IT) can specifically kill subsets of normal or neoplastic cells. The cytotoxicity of A-IT can be increased by addition of immunotoxin containing both the ricin B chain (B-IT) and antibody specific to the A-IT. Presumably, the B chain facilitates the translocation of the A chain (from the internalized A-IT) into the cytoplasm. Previous studies have shown that the binding of B chain to antibody reduces its galactose-binding function so that potentiation induced by B-IT is specific for the determinant to which the antibody is directed. However, such B-ITs can still bind to the galactose-containing glycoprotein, asialofetuin, demonstrating that the B chain retains some lectin activity. It would, therefore, be desirable to eliminate the galactose-binding sites from the ricin B chain. This objective is being approached by cloning the ricin B chain in order to attempt deletion of the galactose-binding sites by site-specific mutagenesis. We have cloned a ricin cDNA in an Okayama-Berg vector and have separated the ricin B chain coding sequences from those of the A chain. The B chain sequences have been subcloned into an expression vector containing the SV-40 early region promotor and a late polyadenylation site. SV-40 transformed monkey kidney cells (COS) have been transfected with this plasmid DNA. The expressed ricin B chain is being detected by biochemical and functional assays.

G41 SPECIFIC CLEAVAGE OF DIPHTHERIA TOXIN BY A PLASMINOGEN ACTIVATOR, Witold Cieplak and Leon Eidels, University of Texas Health Science Center, Dallas, TX 75235

Diphtheria toxin (DT) is a single chain, M_r 60,000 protein synthesized by certain lysogenized strains of Corynebacterium diphtheriae. DT intoxicates eukaryotic cells through binding to specific cell surface receptors, followed by internalization and enzymatic inactivation of elongation factor 2, thereby inhibiting protein synthesis. The binding and enzymatic (ADP-ribosyl transferase) activities have been localized to distinct fragments derived through limited trypsinolysis, termed B (M_r 39,000) and A (M_r 21,000), respectively. This latter finding has led to the proposal that limited proteolysis ("nicking") of the toxin molecule is requisite to the intoxication process and that a trypsin-like protease is involved in this process. To date, no cellular proteases which would fulfill this function have been identified. We have, however, recently found that the human urinary plasminogen activator, urokinase (UK; E.C. 3.4.21.32) is capable of cleaving DT to yield exclusively two fragments which co-migrate in SDS-PAGE with "native" A and B fragments found in crude preparations of toxin. The cleavage pattern produced by UK, however, appears to be similar to, but not identical to, that produced by trypsin treatment in that other degradation fragments are not found. These observations, coupled with the documented widespread distribution of UK-like enzymes in tissues, suggest a possible role for the enzyme in DT-mediated intoxication in vitro and in vivo as well as providing an alternative and perhaps more specific method for the preparation of active A and B fragments.

G42 C9-MEDIATED BACTERIAL KILLING IN THE ABSENCE OF C5b-8, John R. Dankert and Alfred F. Esser, Laboratory for Structural Biology, Department of Comparative & Experimental Pathology, University of Florida, Gainesville, FL 32610.

The molecular mechanism of complement (C)-mediated killing of Gram-negative bacteria has yet to be resolved but it is generally accepted that assembly of the membrane attack complex (MAC) on the outer bacterial membrane is a required step and that the inner membrane (IM) is the ultimate target. We have now investigated the effect of the MAC proteins on the membrane potential (E_m) across the inner bacterial membrane. Since E_m is generated across the IM in Gram-negative bacteria, IM vesicles were prepared and membrane potentials were generated. C9 added in the absence of earlier acting complement proteins had no effect on E_m of isolated, actively respiring vesicles. In contrast, its C-terminal thrombin fragment (C9b) efficiently collapsed E_m in such vesicles. Neither C9 nor C9b alone affected viability of whole cells. However, when C9 gained access to the periplasmic space of transiently permeable and osmotically shocked cells a decrease in cell viability was observed. Omission of C9 during plasmolysis or addition of C9 after plasmolysis did not lead to cell death. We estimate that as few as 8 C9 molecules in the periplasmic space constitute a lethal unit. Since C9 did not dissipate E_m of isolated IM vesicles, we propose that C9 must be processed during entry into the cell envelope to express lethal activity. These results indicate that in vivo C5b-8 serves as a receptor and mediator for C9 entry into the periplasmic space. Thus, bacterial resistance to C killing may arise from inhibition of C9 binding, or C9 entry, or C9 processing. For example, we found that formation of protease-resistant poly-C9 enhances bacterial survival. (Supported by NSF Grant PCM-82 42116 and NIH Grant RO1 AI 19478).

G43 LMTK$^-$ CELLS RESISTANT TO A TRANSFERRIN-DIPHTHERIA TOXIN CONJUGATE, Rockford K. Draper and Donald O. O'Keefe, University of Texas at Dallas, Richardson, TX 75080

The iron-carrying protein transferrin (Tf) delivers iron to mammalian cells by a cyclic process involving receptor-mediated endocytosis. We recently described a cytotoxic form of Tf, prepared by conjugating Tf to diphtheria toxin, that killed mouse LMTK$^-$ cells following endocytosis of the conjugate (Tf-DT) via Tf receptors [O'Keefe and Draper (1985) J. Biol. Chem. 260:932]. We have used Tf-DT to isolate toxin-resistant variants of LMTK$^-$ cells and we are analyzing the variants to determine which have defects in the Tf cycle. We have characterized one such mutant, termed AF 192, in further detail. AF 192 cells are 5-fold resistant to Tf-DT and highly resistant to Pseudomonas aeruginosa exotoxin A and the plant toxin modeccin. AF 192 cells accumulate iron from diferric Tf at one-third the rate of normal cells and this depressed iron uptake cannot be readily explained by differences between normal and variant cells in the binding and internalization of diferric Tf. The properties of AF 192 cells might be explained by a defect in endosomal acidification, which would impair iron release from Tf and also result in toxin resistance; however, we have been unable as yet to detect a defect in vacuolar acidification in extracts from AF 192 cells. We are presently exploring the possibility that initial endocytic vesicles from this variant cell line are not acidic and do not fuse with endosomes, so that endocytosed material is not exposed to a low pH, even though endosomes capable of acidifying their interior may be present.

G44 COMPLEMENT ACTIVITY DEPLETION LIMITS THE IN VITRO KILLING OF CULTURED NEUROBLASTOMA CELLS WITH MONOCLONAL ANTIBODY AND COMPLEMENT, Reggie Duerst and Christopher Frantz, University of Rochester School of Medicine, Rochester, NY 14642

An extensive evaluation was performed of the variables affecting the ability to kill cultured human neuroblastoma (SK-N-MC cell line) cells in vitro with the murine IgG2a anti-neuroblastoma monoclonal antibody (McAb) 6-19 and baby rabbit complement (C'). SK-N-MC cells were prestained with the fluorescent dye Hoechst 33342, which in conjunction with trypan blue, enables rapid, sensitive detection of surviving cells. Although with appropriate treatment conditions greater than 5 logs of cell kill was obtained, C' activity was found to be the factor limiting the extent of cell kill obtainable. With McAb 6-19 at a concentration which gave maximal killing (5-10 μg/ml), cell killing increased proportional to the C' concentration. Prior incubation of C' with McAb and SK-N-MC cells resulted in reduced C' activity primarily from depletion of C' activity and to a much lesser degree from factors that inhibited freshly added C'. The reduction of C' activity was related to the target cell concentration, resulting in less proportional cell kill at higher cell concentrations. The C' activity also decreased as the duration of incubation with McAb and neuroblastoma cells increased. As a result, multiple brief incubations are more effective than a single incubation of the same total duration. This evaluation may prove useful to optimize cell killing with other monoclonal antibody-target cell systems. To simulate tumor cell purging prior to autologous bone marrow transplantation, similar techniques will be used to examine tumor cell killing in the presence of bone marrow cells.

G45 DIPHTHERIA TOXIN: A PROTEIN KINASE WITH AUTOPHOSPHORYLATING ACTIVITY, Leon Eidels and Kyle W. Hranitzky, Department of Microbiology, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235

Diphtheria toxin (M_r 58,342) is a proenzyme possessing ADP-ribosyl transferase activity. Upon limited proteolysis and reduction, its A fragment (M_r 21,167) catalyzes the transfer of ADP-ribose from NAD to elongation factor 2; in the absence of elongation factor 2 this activity is expressed as an NAD-glycohydrolase. We report here a hitherto undescribed third enzymatic activity associated with this bacterial toxin - a protein kinase activity. Incubation of diphtheria toxin with [γ^{32}P]ATP resulted in phosphorylation of the toxin. This kinase activity was associated with the form of toxin with an available nucleotide-binding site (nucleotide-free toxin) and was absent in the toxin molecules with an unavailable nucleotide-binding site (nucleotide-bound toxin). Incorporation of ^{32}P-phosphate from [γ^{32}P]ATP was inhibited by adenylyl-(3',5')-uridine 3'-monophosphate (ApUp), inositol hexaphosphate, NAD, and ATP, ligands that bind to the toxin's nucleotide-binding site. Pretreatment of the nucleotide-free toxin with alkaline phosphatase greatly enhanced the autophosphorylating activity from a level of ∿0.01 moles phosphate incorporated/mole of toxin to a value of ∿0.15-0.2. The ^{32}P-phosphate was present as phosphoserine, as determined by limited acid hydrolysis of the phosphorylated toxin and analysis by high voltage paper electrophoresis. Taken together, these results strongly suggest that diphtheria toxin is a bacterial phosphoprotein with protein kinase activity.

G46 THE ENZYMIC ACTIVITY OF CHOLERA TOXIN REQUIRES A GTP-BINDING PROTEIN OF THE MEMBRANE. D. Michael Gill, Jenifer Coburn, and Amichai Tamir, Tufts University School of Medicine, Boston, Massachusetts 02111.

Cholera toxin requires GTP for its activity. It used to be thought that the GTP bound to toxin substrates, primarily the adenylate cyclase regulator Ns. It is now clear that the relevant GTP binds to a distinct membrane protein that we have called "S," and that successful binding of the GTP requires another, soluble protein, "CF" (19,000 Mr). Current indications are that CF may catalyse a GTP for GDP exchange on S, much as EF-Ts acts to generate EF-Tu[GTP] from EF-Tu[GDP]. Activated S (presumably S[GTP]) stimulates the ADP-ribosyl transferase activity of cholera toxin towards major and minor protein substrates, but not the NADase or ADP-ribosyl-arginine transferase activities. Activated S can be removed from the membrane and retains its activity in detergent solution. The protein substrates do not all bind GTP. These conclusions apply equally to erythrocyte and brain membranes and are probably generally applicable. In the erythrocyte at least, the major substrate (N_S) seems to be most readily ADP-ribosylated when it is free of guanylnucleotide and therefore inactive.

G47 TEMPERATURE-DEPENDENT DIPHTHERIA TOXIN CHANNELS PROVIDE EVIDENCE FOR COORDINATION BETWEEN FRAGMENT A INSERTION INTO MEMBRANES AND CHANNEL FORMATION. Valerie W. Hu, Uniformed Services University of the Health Sciences, Bethesda, MD. 20814-4799

Diphtheria toxin channels have been studied by following the release of a fluorescent dye encapsulated within large unilamellar vesicles as a function of pH and temperature. Dye release shows a strong pH dependence, markedly increasing at pH 5.5. Below pH 5.5, both the extent and rate of marker release increase such that the half-time for dye release is on the order of seconds (or less) at pH 4.0. While these toxin channels are active at room temperature, they are significantly suppressed at ~0 °C. Our previous studies with a membrane-restricted photoprobe have indicated that, at room temperature, both fragments A and B are inserted into these model membranes but that, at 0 °C, the insertion of fragment A, but not B, is restricted(J. Biol. Chem.(1984)259:12226). The present data indicate that insertion of fragment A into membranes is correlatable with toxin channel formation and that fragment B insertion *per se* (i.e., at 0 °C) does not necessarily lead to active channels, implying that a temperature-dependent *post-insertional* step is required for channel formation by diphtheria toxin. These results do not necessitate fragment A passage through the fragment B channel but suggest that, for native toxin, the processes of fragment A insertion and channel formation are at least coordinated.

G48 TOXIN TRANSLOCATION TO THE CYTOPLASM: MECHANISMS FOR SINGLE-HIT KINETICS. Thomas H. Hudson and David M. Neville, Jr., Laboratory of Molecular Biology, NIMH, Bethesda, MD 20892

Protein synthesis inhibition by diphtheria toxin (DT), ricin (R), *Pseudomonas* exotoxin-A (PE) or Modeccin (M) displays first order kinetics after an initial dose-dependent lag period. The inhibitory effects are the result of (1) toxin binding to receptors, (2) translocation of toxin into the cytosol, and (3) biochemical inactivation of the protein synthesizing machinery. In the case of DT, the translocation of A chain to the cytosol has been shown to be the rate limiting step responsible for the single-hit process (Hudson & Neville, JBC 260: 2675). Autoradiographic analysis of intoxicated cell populations support this conclusion with the finding of two subpopulations; one composed of cells with normal rates of protein synthesis and the other composed of non-synthesizing cells. As intoxication progresses the normal cells rapidly convert to non-synthesizing cells. Autoradiographic analysis of R, PE, and M intoxicated cell populations reveal the same pattern suggesting that, early in intoxication (during the lag) each cell is accorded an equal probability of receiving to its cytoplasm an amount of toxin sufficient to rapidly (within minutes) block all of its protein synthesis. After the probability is established, the toxin translocation event can be blocked by 2-deoxy-D-glucose and azide as well as incubation in the cold. The energy required is not utilized in vesicle acidification as this necessary step in DT intoxication occurs during the lag. Neither oligomycin nor dinitrophenol has an effect on the translocation process indicating that a proton motive force across the vesicle membrane is not a requirement.

G49 RIBOSOME-INACTIVATING PROTEINS FROM THE SEEDS AND LEAVES OF SAPONARIA OFFICINALIS, D.A. Lappi, P.C. Montecucchi, D. Lazzarini, D. Martineau, T. Cadeddu and M. Soria, Farmitalia Carlo Erba SpA, Milan, Italy.

Several different ribosome-inactivating proteins (RIPs) are contained in the seed extracts of Saponaria officinalis. Carboxymethyl cellulose chromatography of the extracts reveals several peaks, in which over 80% of the activity is recovered in peaks 5 (SO-5), 6 (SO-6), 8 (SO-8) and 9 (SO-9) (Stirpe, et al., 1983, Biochem, J. 216:617-625). Recently one of these, SO-6, has been conjugated to a monoclonal anti-Thy 1.1 antibody and the resulting immunotoxin shows potent anti-tumor cell activity in vivo in nude mice bearing peritoneal AKR-A lymphoma cells (Thorpe, et al., 1985, JNCI 75:151-159).

We have chromatographed each individual peak on reverse phase chromatography (RPC) and find a family of proteins with similar properties with respect to structure. Six proteins were distinguished that have similar physical properties according to the following criteria: high pI, similar molecular weight, similar N-terminal amino acid sequence and immunocross-reactivity.

The leaves of Saponaria also contain RIP activity. Using the antisera raised against SO-6 as an affinity ligand, we have purified the major RIP species from the leaves and identified it as SO-5 by co-chromatography on FPLC non-exchange. SO-5 from leaves has physical properties similar to SO-5 from seeds.

G50 STRUCTURE AND ACTIVITY OF PARDAXIN, A NEW PRESYNAPTIC TOXIN, IN ARTIFICIAL LIPID MEMBRANES, Philip Lazarovici[1], Naftali Primor[2], Carlo G. Caratsch[3], and Leslie M. Loew[4], [1]Weizmann Inst. of Science, Rehovot, Israel, [2]Osborn Lab. of Marine Sciences, NY, USA, [3]Univ. of Zurich, Zurich, Switzerland, [4]Univ. of Connecticut, Farmington, USA.

Using chromatofocusing, a new presynaptic toxin, designated Pardaxin, was purified from the gland secretion of the flatfish Pardachirus marmoratus. This toxin increases the frequency of the spontaneous release of transmitter quanta up to more than 100 times on frog skeletal neuromuscular junction. Pardaxin was characterized as a single chain, acidic protein, composed of 120 amino acids, free of carbohydrate, lipid,sialic acid or sterol residues. It is an amphiphatic, hydrophobic protein containing a compact hydrophobic domain on the N-terminal (NH_2-Gly-Phe-Phe-Ala-Leu-Ile-Pro-Gly-Ile-Glu) and which tends to aggregate in aqueous solutions. In planar artificial membranes, under voltage clamp conditions, Pardaxin increases the electrical conductence, as a result of formation of defined pores, which open only when the applied voltage is positive with the respect to the trans side of the membrane. In hyperpolarized unilamellar liposomes and nultilamellar liposomes with an imposed chemical gradient, Pardaxin produced a fast ion nonspecific and a slow cation specific pore, respectively. ^{125}I-Pardaxin binds and is sequestered by liposomes and its insertion into the bilayer was revealed by hydrophobic photolabeling of liposomes displaying pore activity. It is suggested that Pardaxin exert its presynaptic effects due to its hydrophobicity and pore formation properties. *This work was supported by a research grant from the Department of Defence, Office of Naval Research, U.S. Navy.

G51 THE ROLE OF LYSOSOMAL ENZYMES IN CELL KILLING BY THE LYSOSOMOTROPIC DETERGENT DODECYL IMIDAZOLE. P. Wilson, R. Firestone*, R. VanDeroef and J. Lenard. Dept. Physiol., UMDNJ-R.M.S., Piscatway, NJ. *Merck, Sharp & Dohme, Rahway, N.J.

Lysosomotropic detergents (LDs) are amines with pK's of 5-9 possessing aliphatic hydrocarbon "tails" of ca. 12 carbons, which kill cells with a sigmoidal dose dependence. Previous evidence (JCB 97 1841-51, 1983) supports the following mechanism for cell killing: (i) LDs diffuse across cell membranes in the uncharged form; (ii) they become protonated (charged) and accumulate in lysosomes and other acidic organelles; (iii) upon exceeding some critical concentration, they acquire detergent properties, damaging the lysosomal membrane and permitting hydrolytic enzymes to escape into the cytoplasm, which kills the cell. The role of lysosomal enzymes in cell killing by the LD dodecyl imidazole (C_{12}-Im) was tested in two ways. First, I-cell disease fibroblasts, which possess about 15% of normal levels of mannose-6-phosphate containing lysosomal enzymes, were compared with normal human fibroblasts. Normal fibroblasts were about two times more sensitive than I-cell fibroblasts to killing by C_{12}-Im. Second, normal CHO fibroblasts were depleted of mannose-6-phosphate containing lysosomal enzymes by growth in 10 mM NH_4Cl, which causes secretion of lysosomal enzymes. Growth in this medium was normal, but after 3 ½ days cellular β-hexosaminidase activity was 12-25% of normal. Sensitivity to C_{12}-imidazole was significantly reduced in these cells, but returned to control levels after removal of NH_4Cl and recovery of normal lysosomal enzyme levels. We conclude that mannose-6-phosphate containing lysosomal enzymes are integral to the mechanism of cell killing by LDs, as postulated above, and that lysosomal enzymes may act as toxic proteins in the cytoplasm.

G52 CLONED cDNA CODING FOR PREPRORICIN

J. Michael Lord and Lynne M. Roberts, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

Naturally occurring cytotoxic proteins such as ricin would have improved pharmacological potential if some of their normal functional properties could be effectively altered. Modification of the DNA encoding the toxin polypeptides is the most obvious route to this end. To permit these manipulations we have deduced the primary structure of a precursor protein that contains the toxic (A) and cell-binding (B) chains of ricin from the nucleotide sequence of cloned cDNA. Preproricin mRNA encodes a single polypeptide containing both the A and B chain sequences. An N-terminal signal sequence (probably 15 amino acids in length) precedes the A chain sequence which is itself separated from the B chain sequence by a 12 amino acid long linking segment. *In vivo* precursor processing occurs post-translationally in the protein bodies where an acid endoprotease excises the linking segment and may also involve cleavage of a 9 residue segment from the N-terminus of the A chain. Preproricin cDNA clones have been distinguished from the largely homologous prepro*Ricinus communis* agglutinin (RCA) clones by restriction site analysis and by direct comparison of part of the deduced RCA B chain sequence with that obtained by direct protein sequencing of purified RCA B chain.

G53 ISOLATION AND CHARACTERIZATION OF A CYTOLYTIC, MEMBRANE PORE-FORMING PROTEIN (PERFORIN) FROM THE PATHOGENIC AMOEBA, *Naegleria fowleri*.

David Lowrey and Eckhard R. Podack, New York Medical College, Valhalla, NY

Naegleria fowleri, the etiological agent of fatal primary amoebic meningoencephalitis, is endowed with a potent cytolytic activity that may be related to the pathogenesis of this disease. We have purified a potent cytolytic protein (N-perforin) from the plasma membrane of amoebae by differential centrifugation followed by detergent extraction and FPLC-Superose 12 gel filtration chromatography. N-perforin occurs in two cytolytically active forms. In the absence of detergent it elutes as a monomer of M_r 70,000. In the presence of Zwittergent 3-12 micelles it oligomerized to a complex with an apparent M_r of 7-10 x 10^5. Oligomerization was time and tempature dependent and required detergent micelles. SDS-PAGE analysis of the two species of N-perforin revealed that both consist of a single polypeptide with an M_r of 66,000. Under non-reducing conditions, disulfide-linked oligomers at M_r 125,000 and greater than 200,000 as well as a 56,000 M_r monomer were present in both species. Examination of erythrocyte membranes lysed by the purified N-perforin revealed pore complexes of 15-16 nm outer diameter and 3-4 nm inner diameter. N-perforin appears to be homologous to perforin 1 of cytotoxic lymphocytes as well as the ninth complement component with regard to molecular weight, disulfide linkages, and polymerization into cytolytic pores.

G54 Activation of haemolysin 2001 from E.coli requires a post-translational modification promoted by HlyC

N. Mackman, J.-M. Nicaud, L. Gray & I.B. Holland, University of Leicester.

The haemolysin encoded by the wild type *E.coli* strain 2001 (O4 serotype) isolated from a urinary tract infection is secreted as a 107 Kd protein to the medium. The structural gene for this polypeptide is *hlyA* but insertional mutagenesis by Tn5 has shown that a second gene, *hlyC* is also required for activity of the haemolysin. In the complete absence of the *hlyC* gene which encodes a 20 Kd cytoplasmic protein, the 107 Kd protein is still secreted to the medium but it is inactive. The inactive protein is more stable to degradation at 37° and more resistant to digestion by various proteases including trypsin. In addition, the inactive form migrates with a reduced mobility in non-denaturing acrylamide gels. These results indicate that HlyC promotes a post translational modification of HlyA which is probably essential for binding of the 107 Kd protein to the red cell surface and/or for its subsequent insertion into the surface to form a specific pore leading to lysis of the erythrocytes.

G55 MECHANISMS OF IN VITRO NEUTRALIZATION OF ALPHAVIRUSES BY MONOCLONAL ANTIBODIES
James H. Mathews and John T. Roehrig, Division of Vector-Borne Viral Diseases, Centers for Disease Control, Fort Collins, CO 80526

We have previously identified at least eight epitopes on the E2 glycoprotein of Venezuelan equine encephalomyelitis (VEE) virus using monoclonal antibodies (MAbs). Some of these antibodies identified a critical neutralization (N)-site. Passive transfer of MAbs reactive with this site were efficient at protecting animals from a lethal viral encephalitis. Antibody fragmentation studies determined that an intact Fc was required for in vivo protection, and bivalent antibody was required for in vitro virus N. We are currently characterizing virus-cell interactions, and the mechanisms by which neutralizing MAbs inhibit virus replication in vitro. Adsorption studies were performed with radioactively labelled VEE virus on Vero and human diploid cells. Dilutions of homologous and closely related heterologous viruses could compete for binding with radioactive marker virus for adsorption in a typical dose-response relationship. Incubation of virus with various preparations of MAbs prior to adsorption, in conjunction with monitoring viral RNA synthesis, suggested three related mechanisms of virus N. Ten µg of MAbs which define the critical N-site inhibits 90% of a 5 µg (1×10^7 pfu) dose of virus from binding to cells. MAbs which define epitopes spatially proximal to the critical N-site (with low but demonstrable N titers), are less efficient at blocking (50-60% inhibition), but equally efficient at suppressing a productive infection. A third mechanism involves a MAb which enhances binding of virus to cells, however the infection is significantly inhibited.

G56 MECHANISMS OF RESISTANCE AND RECOVERY OF NUCLEATED CELLS SUBJECTED TO COMPLEMENT ATTACK,
B.P. Morgan*#, J.R. Dankert# and A.F. Esser#, *University of Wales College of Medicine, Cardiff, U.K. and #Department of Comparative & Experimental Pathology, University of Florida, Gainesville, FL 32610.
Nucleated cells can resist and recover from complement (C) attack even after formation of the cytolytic membrane attack complex (MAC) on the cell surface. In human neutrophils (PMN) a major mechanism of resistance is the rapid elimination of MACs by exocytosis. More than 60% of the bound MAC proteins are removed in the form of membrane vesicles constituting only 2% of the cell surface. A secondary mechanism of elimination in PMN is endocytosis of MAC and degradation of C proteins within the cell. Cell death is enhanced through inhibition of vesiculation by: (i) removal of extracellular Ca^{2+}; (ii) incubation with metabolic inhibitors; and (iii) prevention of poly-C9 formation.
After sublytic C attack PMN are metabolically depleted as a result of energy expenditure during recovery. However, incubation of cells in nutrient buffer rapidly restores cellular functions as demonstrated by measurements of cAMP levels, O_2 radical production, phagocytosis of immune complexes, and chemotaxis. These results demonstrate that in human PMN subjected to sublytic C attack, recovery processes rapidly and completely restore normal cell function. (Supported by NIH Grant RO1-AI 19478 and the MRC).

G57 BIOCHEMICAL, ANTIGENIC AND FUNCTIONAL PROPERTIES OF THE PURIFIED EPSTEIN-BARR VIRUS/C3d RECEPTOR OF HUMAN B LYMPHOCYTES.
Glen R. Nemerow, Martin F.E. Siaw, Carolyn Mold and Neil R. Cooper, Scripps Clinic & Research Foundation, La Jolla, CA. 92037.
Previous studies showed that a B cell specific monoclonal antibody (MoAb), termed OKB7, recognized a 145 kDa protein which directly blocked binding and infection of human B cells by Epstein-Barr virus (EBV) and in addition inhibited C3d receptor (CR2) function. In these studies the 145 kDa protein was analyzed by immunoprecipitation with four anti-CR2 MoAbs which recognize non-overlapping epitopes. A single membrane glycoprotein of 145 kDa was observed with no discernable heterogeneity as assessed by protease and endoglycosidase mapping studies. An efficient purification procedure using immunoaffinity chromatography was developed for the isolation of the 145 kDa protein from Raji B lymphoblastoid cells. The protein was recovered with a yield of 75% with purity in excess of 95% as determined by densitometric scanning of silver stained SDS-gels. An amino acid compositional analysis of the purified protein revealed a high content of serine, glycine and cysteine residues while the content of the other amino acids was unremarkable. An ELISA showed that the purified protein reacted with each of the anti-CR2 monoclonal antibodies. The purified protein was incorporated into ^{14}C-phosphotidylcholine liposomes in an external orientation as demonstrated by immunoelectron microscopy. These unilamellar vesicles containing the 145 kDa protein were shown to bind to sheep red blood cells bearing C3d and to purified EBV and to EBV infected cells. Both of these ligand binding properties were blocked by the OKB7 MoAb. These studies demonstrate conclusively the dual functional activity of the EBV/C3d receptor and provide the basis for further studies on the molecular biology of this membrane receptor.

G58 DIFFERENTIAL SENSITIVITY OF TRYPANOSOMES TO HDL-INDUCED LYSIS. M.R. Rifkin and F.R. Landsberger, The Rockefeller University, New York, NY 10021.
Trypanosomes are parasitic protozoa that appear in the bloodstream of infected mammalian hosts in different morphological forms. Serum from non-permissive hosts is cytotoxic for these organisms and leads to cell lysis, slender trypanosomes being more susceptible to lysis than stumpy forms. The serum trypanocidal factor has been identified as high density lipoprotein (HDL) and causes cell lysis by acute, irreversible damage to the permeability properties of the cell's plasma membrane. To asses whether trypanosome lysis by HDL is due to specific membrane properties, such as observed lipid bilayer fluidity, ESR spin label studies were performed using 5-doxyl-stearic acid (C_5). The C_5 ESR spectral splitting did not reveal any differences between slender and stumpy trypanosomes or betweeen strains of trypansomes differing in their sensitivity to lysis. The trypanosome major surface glycoprotein is anchored in the membrane via covalently linked myristic acid containing phosphatidylinositol. This membrane protein might induce or alter lateral lipid domain structure and so induce bilayer lateral inhomogeneity; different proteins might vary in the strength of their interactions with the bilayer and hence create sharper interphases which would lead to greater instabilities and thus enhance susceptibility of the trypanosome to lysis. These considerations lead to a postulated structural model of glycoprotein-induced lateral inhomogeneity which leads to the prediction that acidic lipid distribution in the plasma membrane of slender and stumpy trypanosomes may determine sensitivity to HDL-induced lysis. (Supported by NIH grant AI-20324 and NSF grant PCM8409213)

G59 CLONED cDNA CODING FOR PREPRORICIN
J. Michael Lord and Lynne M. Roberts, Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

Naturally occurring cytotoxic proteins such as ricin would have improved pharmacological potential if some of their normal functional properties could be effectively altered. Modifications of the DNA encoding the toxin polypeptides is the most obvious route to this end. To permit these manipulations we have deduced the primary structure of a precursor protein that contains the toxic (A) and cell-binding (B) chains of ricin from the nucleotide sequence of cloned cDNA. Preproricin mRNA encodes a single polypeptide containing both the A and B chain sequences. An N-terminal signal sequence (probably 15 amino acids in length) precedes the A chain sequence which is itself separated from the B chain sequence by a 12 amino acid long linking segment. *In vivo* precursor processing occurs post-translationally in the protein bodies where an acid endoprotease excises the linking segment and may also involve cleavage of a 9 residue segment from the N-terminus of the A chain. Preproricin cDNA clones have been distinguished from the largely homologous prepro*Ricinus communis* agglutinin (RCA) clones by restriction site analysis and by direct comparison of part of the deduced RCA B chain sequence with that obtained by direct protein sequencing of purified RCA B chain.

G60 SENSITIVITY AND RESISTANCE TO DIPTHERIA TOXIN, RICIN AND MODECCIN IN CHINESE HAMSTER OVARY CELL MUTANTS DEFECTIVE IN INTRACELLULAR PROCESSING OF METHOTREXATE POLY(LYSINE), Hugues J.P. Ryser, Richard Mandel, and Asteghik Hacobian, Boston University School of Medicine, Boston, MA, 02118.

Mutants of Chinese hamster ovary cells defective in endocytosis were selected on the basis of their resistance to a poly(lysine) conjugate of methotrexate (MTX-poly(Lys) and of their normal sensitivity to the free drug. Two of them, $MPL^R(3\text{-}4)$ and $MPL^R(2\text{-}5)$, were found to be cross resistant to diptheria toxin (DTX) and modeccin, and hypersensitive to ricin. When this hypersensitivity was used to select revertants of $MPL^R(3\text{-}4)$, one of the revertants ($R^S5\text{-}1$) was still sensitive to DTX, while another ($R^S4\text{-}3$) was normally sensitive to ricin and DTX, but still resistant to MTX-poly(Lys) and modeccin. In both $MPL^R(3\text{-}4)$ and $MPL^R(3\text{-}4)R^S(4\text{-}3)$, the resistance to MTX-poly(lys) was associated to a 50% reduction of the mutants' ability to degrade internalized ^{3}H-MTX-poly(lys). Hybridization of either mutants with wild type cells corrected the resistance to both MTX-poly(lys) and DTX. These data suggest that the genetic defect in intracellular processing of MTX-poly(lys) that led to the selection of $MPL^R(3\text{-}4)$ is causally related to the mutant's cross resistance to modeccin, but only coincidentally related to its cross resistance to DTX. $MPL^R(3\text{-}4)$ thus appears to be a mutant carrying both an endosomal and a post-endosomal defect, both of which are corrected through complementation, but only one of which was reverted when ricin was used as a selective agent. Since selection in presence of ricin can yield revertants that either keep or lose their DTX resistance, it appears that ricin hypersensitivity and DTX resistance are caused by separate defects. (Supported by NCI-Grant CA-14551)

G61 ENTRY OF PROTEIN TOXINS INTO CELLS, Kirsten Sandvig and Sjur Olsnes, Norsk Hydro's Institute for Cancer Research, Montebello, Oslo, Norway.

A number of protein toxins like abrin, ricin, diphtheria toxin, modeccin and shigella toxin exert their effect on cells after entry into the cytosol. In spite of their structural simularity, their entry mechanisms differ. Diphtheria toxin seems to enter the cytosol from early acidic vesicles, and a rapid entry of cell surface bound toxin can be induced by decreasing the pH in the medium. The requirements for this direct entry has been studied. In addition to low pH, entry into the cytosol requires permable anions and a pH gradient across the membrane. It is possible to divide the entry of diphtheria toxin into two steps, insertion of toxin into the membrane without transport into the cytosol, and a subsequent transport of fragment A into the cell. Insertion of diphtheria toxin into the membrane inhibits anion transport, and it is possible that the anion exchanger in Vero cells functions as the receptor for diphtheria toxin. Also modeccin and possibly also shigella toxin seem to require low pH for entry.

G62 MYOTONIC MUSCULAR DYSTROPHY: ABERRANT ENDOCYTOSIS OF EGF AND α-MANNOSIDASE, Patricia Schimke and Paul Schlesinger, Washington University, St. Louis, MO 63110.
Human skin fibroblasts from apparently normal patients and patients with Myotonic Muscular Dystrophy (MMD) were obtained from the N.I.G.M.S. Human Genetic Mutant Cell Repository, (GM3377A and GM3132) and were employed in the investigation of receptor-mediated endocytosis of α-Mannosidase and EGF. Previous studies of mannose-6-phosphate (M6P) specific endocytosis of α-Mannosidase indicated that it's uptake was decreased in MMD fibroblasts but that the acidification of intracellular vesicles was apparently normal in MMD fibroblasts (JCB, 99, 372a, Oct 1984). α-Mannosidase uptake was characterized by a decrease in the maximal rate of uptake from 5 fM/min (normal) to 0.67 fM/min (MMD) both with a Km of 1.5nM. By scatchard analysis both cell types displayed 11,000 cell surface M6P receptors with an apparent affinity of 1.4 nM. This implied that the endocytic alteration in MMD fibroblasts occurs either subsequent to binding but prior to the intracellular separation of ligand from receptor, or subsequent to intracellular separation but prior to the return of unoccupied receptor to the cell surface. Impaired EGF uptake was observed in MMD fibroblasts, uptake was characterized by a decrease in the maximal rate of uptake from 3.33 fM/min (normal) to 1.4 fM/min (MMD). The Km of uptake was similar in both cell types, 11.8 nM (normal) and 7.8 nM (MMD). The initial rate of uptake at saturation was decreased in MMD fibroblasts. Comparing the characterization of EGF and α-Mannosidase uptake in normal and MMD fibroblasts, it is evident that the alterations between these cell types are similar for both endocytic systems. Imparied uptake of EGF could result from an alteration in the number of cell surface receptors and/or a defect early in the endocytic pathway.

G63 MODIFIED LIPOPROTEINS AS CARRIERS OF LIPOPHILIC PRODRUGS FOR MEMBRANE-DIRECTED INTERACTIONS. J. Michael Shaw*, Kala V. Shaw, Larry H. Matherly, Roopa Baichwal, Charles K. Barlow, Lawrence B. Schook & Andre Rosowski, Virginia Commonwealth Univ., Depts. of Biochemistry, Immunology & Medicine, Richmond, Va. 23298 and Dana Farber Cancer Inst., Boston, Mass. 02115 *Present Address, Alcon Labs., Drug Delivery , Fort Worth, Texas 76134.

Several classes of lipophilic drugs have been partitioned noncovalently into hydrophobic domains of low density lipoprotein (LDL), acetylated-LDL or antibody H_2K^K covalently modified-LDL. The drugs included hexadecylmethotrexate (HMTX) hexadecylarabinofuranosyl-CMP, muramyltripeptide phosphatidylethanolamine (MTP-PE) and the lipophilic anthracycline, AD-32. Following chromatography and filtration, between 40 and 150 molecules of drug/particle were associated with the lipoprotein complex. The drug molecules when complexed to LDL showed good stability with the exception of HMTX which was hydrolyzed to methotrexate with a t 1/2 of 50h at 4°. The LDL-HMTX was inhibitory toward purified dihydrofolate reductase and was cytotoxic toward L-1210 leukemia cells. Acetylated-LDL interacts with cell types possessing a different receptor from the apoprotein B-E receptor. Acetylated-LDL complexed with the immunomodulator, MTP-PE interacts specifically with mouse peritoneal macrophages. The normal apoprotein B-LDL receptor binding affinity also could be altered by the covalent attachment of antibody H_2K^K to oligosaccharide regions of apoprotein B. Anti-H_2K^K-LDL AD-32 complex showed binding to L929 H_2K^K positive cells with negligible interaction to receptor negative HELA and VERO cells. In summary, lipoproteins can serve as carriers of several classes of lipophilic drugs and when suitably modified, lipoproteins interact with varied receptors and membrane regions on different cells types.

G64 AMINO ACID RESIDUES OF RICIN INVOLVED IN GALACTOSE-BINDING, Edward J. Wawrzynczak, Anna Falasca, William A. Jeffery and Philip E. Thorpe, Imperial Cancer Research Fund Laboratories, London, U.K.

The galactose-binding capacity of ricin which mediates the binding of the toxin to cells is reduced by acetylation of tyrosine residues. In common with native ricin, the galactose-binding ability of isolated ricin B-chain (measured by affinity for Sepharose 4B, a galactose-based adsorbent) was reversibly inactivated by acetylation of tyrosine residues. There was a progressive loss of galactose-binding ability with increasing acetylation that correlated with the modification of two tyrosine residues. Lactose protected the B-chain against acetylation of these binding site residues.

In order to identify the regions of the toxin molecule involved with galactose-binding, the B-chain was reacted with radiolabelled N-acetylimidazole in the presence and absence of lactose. The modified proteins were subjected to limited proteolysis and radiolabelled fragments identified after separation by high performance liquid chromatography.

G65 THE ROLE OF LYSOSOMAL ENZYMES IN CELL KILLING BY THE LYSOSOMOTROPIC DETERGENT DODECYL IMIDAZOLE. P. Wilson, R. Firestone*, R. VanDeroef and J. Lenard. Dept. Physiol., UMDNJ-R.M.S., Piscatway, NJ. *Merck, Sharp & Dohme, Rahway, N.J.

Lysosomotropic detergents (LDs) are amines with pK's of 5-9 possessing aliphatic hydrocarbon "tails" of ca. 12 carbons, which kill cells with a sigmoidal dose dependence. Previous evidence (JCB 97 1841-51, 1983) supports the following mechanism for cell killing: (i) LDs diffuse across cell membranes in the uncharged form; (ii) they become protonated (charged) and accumulate in lysosomes and other acidic organelles; (iii) upon exceeding some critical concentration, they acquire detergent properties, damaging the lysosomal membrane and permitting hydrolytic enzymes to escape into the cytoplasm, which kills the cell. The role of lysosomal enzymes in cell killing by the LD dodecyl imidazole (C_{12}-Im) was tested in two ways. First, I-cell disease fibroblasts, which possess about 15% of normal levels of mannose-6-phosphate containing lysosomal enzymes, were compared with normal human fibroblasts. Normal fibroblasts were about two times more sensitive than I-cell fibroblasts to killing by C_{12}-Im. Second, normal CHO fibroblasts were depleted of mannose-6-phosphate containing lysosomal enzymes by growth in 10 mM NH_4Cl, which causes secretion of lysosomal enzymes. Growth in this medium was normal, but after 3 ½ days cellular β-hexosaminidase activity was 12-25% of normal. Sensitivity to C_{12}-imidazole was significantly reduced in these cells, but returned to control levels after removal of NH_4Cl and recovery of normal lysosomal enzyme levels. We conclude that mannose-6-phosphate containing lysosomal enzymes are integral to the mechanism of cell killing by LDs, as postulated above, and that lysosomal enzymes may act as toxic proteins in the cytoplasm.

Colicins and Yeast Toxins

G66 Mode of action of E.coli haemolysis 2001

S. Bhakdi*, N. Mackman, J.-M. Nicaud & I.B. Holland
*University of Giessen, and University of Leicester

Haemolysin 2001 was purified as a 107 Kd polypeptide secreted to the medium by E.coli MC4100 (pLG570). The toxin binds to the surface of red blood cells promoting the influx of $^{45}Ca^{2+}$ and the rapid efflux of cellular K^+ independently of cell lysis. From the size of marker molecules such as Dextran 4 in these studies we conclude that the toxin creates pores in the red cell surface of about 3 mm diameter. In contrast to the pore-forming toxins of gram-positive organisms such as streptolysin-O the active haemolysin appears to be a monomer. The addition of trypsin to toxin treated cells failed to affect the specific permeability changes induced by haemolysis and resulted in the protection of two approximately 80 Kd polypeptides. We conclude therefore that the active molecule is inserted into the red cell surface forming a trans-membrane structure.

Immunotoxins

G67 BLOOD CLEARANCE AND TISSUE LOCALISATION OF RICIN A-CHAIN AND DEGLYCOSYLATED-RICIN A-CHAIN IMMUNOTOXINS, David C. Blakey, Graham J. Watson, Phillip Knowles and Philip E. Thorpe, Imperial Cancer Research Fund Laboratories, London, U.K.

The effectiveness of antibody-ricin A-chain conjugates in vivo might be limited by their rapid clearance by hepatic reticuloendothelial cells which have receptors that bind the mannose-terminating oligosaccharides present on the A-chain. Chemical deglycosylation of ricin A-chain reduced its uptake by hepatic Kupffer cells in vitro by 95% and in the liver of mice in vivo by 60-70%. Ricin A-chain and deglycosylated ricin A-chain conjugates were prepared using the monoclonal anti-Thy1.1 antibody and 2-iminothiolane coupling agent. The ricin A-chain immunotoxin was rapidly removed from the bloodstream of mice, only 10% of the injected immunotoxin remained in the bloodstream 3 hours after intravenous administration. The major site of clearance was the liver, approximately 30% of the injected dose localising in this tissue within 10 minutes. The deglycosylated ricin A-chain conjugate was cleared less rapidly, 10% of the injected dose still remaining in the bloodstream after 16 hours and liver uptake was greatly diminished. Clearance of the deglycosylated ricin A-chain conjugate was almost identical to that of immunotoxins made with abrin A-chain which does not have carbohydrate side chains. These results suggest that deglycosylation of ricin A-chains eliminates clearance mediated via its carbohydrate side chains.

G68 **A human adenocarcinoma specific monoclonal antibody, directed to Y carbohydrate antigen mediate cytotoxic activity for tumor cells in vitro and in vivo systems.** Magdalena Blaszczyk, Jan Thurin, Yasuhiko Kimoto, Michael Lubeck, *Ole Hindsgaul, Zenon Steplewski and Hilary Koprowski. The Wistar Institute, Philadelphia, PA 19104, *Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada.

A mouse monoclonal antibody, BR 55-2, was specific for human gastrointestinal tract and breast adenocarcinomas. The antigenic determinant was represented with the Y carbohydrate structure on glycolipid in the GI tumor cell lines and on glycoprotein in human breast adenocarcinoma cell lines. The efficiency of in vivo protection of tumor growth in nude mice is higher in the cells carrying the glycolipid form of the antigen. This observation provide an exellent model for studies on the antibody-mediated cytotoxicity mechanism of tumor cells and particularly the role of tumor associated antigens.

G69 BORONATED MONOCLONAL ANTIBODY CONJUGATES FOR NEUTRON CAPTURE THERAPY (NCT), D.C.Borg J.Elmore, D.Gabel[1], R.Fairchild and S.Ferrone[2], Brookhaven Nat. Lab., Upton NY 11973

The $^{10}B(n,\alpha)^{7}Li$ reaction for NCT optimizes antibody(AB)-directed radiotherapy: 1) The He and Li are high LET particles that provide maximum therapeutic gain, 2) their short ranges (<10μ) can capitalize on cell-specific AB localization, and 3) unlike the case with other modes of radio-immunotherapy, neutron activation can await desired target:background ratios (hours to days after AB administration) without cumulative toxicity. We have conjugated monoclonal AB's (MAB) against human melanoma with the several hundred ^{10}B atoms/MAB required for NCT. Specific MAB activity was retained by using dextran intermediate carriers. Competitive binding assays with human cell lines show specific uptake of ^{10}B in melanoma cells of 8:1 or more over co-incubated nonmelanoma cells and with concentrations (10-30 μg/g) within the range required in target tissues for successful NCT. The sensitivity to inactivation by our chemical modifications varied among the five MAB's tested.

Studies of ^{10}B distribution in melanomas implanted in athymic mice and of enhanced radiation killing of cultured melanoma cell lines following neutron irradiation were scheduled as the abstract was written, but the results already in hand appeared to be the most promising achieved thus far for targeted NCT. Improved procedures for conjugating MAB's with ^{10}B compounds were also under development.

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G70 STUDY OF THE PLASMA CLEARANCE OF ANTIBODY-RICIN A-CHAIN IMMUNOTOXINS EVIDENCE FOR SPECIFIC RECOGNITION SITES ON THE A-CHAIN THAT MEDIATE RAPID CLEARANCE OF THE IMMUNOTOXIN, Pierre Casellas, Bernard Bourrié, Hildur E. Blythman and Franz K. Jansen, Centre de recherches Clin Midy Sanofi, Rue J. Blayac, 34082 Montpellier Cedex. France.

To achieve "in vivo" therapy with immunotoxins it is necessary that these molecules remain in circulation at a sufficiently high level for a sufficiently long time to allow binding to tumor cells to occur. Therefore, examination of the pharmacology of immunotoxins may elucidate the reasons for the poor "in vivo" tumoridical effect of immunotoxin described before. In this study the plasma clearance of antibody-ricin A-chain immunotoxins, after intravenous injection in animals of different species, has been examined. Sensitive and reproducible techniques were developed to monitor the level of immunotoxin and its constituents in the blood. It is shown that immunotoxins are rapidly eliminated from the bloodstream. Neither the properties of the antibody moiety nor the nature of the linkage binding ricin A-chain to antibody account for the disappearance of immunotoxin from the plasma. On the other hand we found that the rapid clearance of immunotoxin is due to the mannose residues on the ricin A-chain moiety which are specifically recognized by liver cells. When immunotoxin is administrated together with yeast mannan, which enhances the level of active immunotoxin in circulation by inhibition of liver uptake, the anti-cancer efficacy of immunotoxin "in vivo" is drastically improved. This demonstrates that the normal pharmacology of immunotoxin acts as a strong antagonist for the full expression of immunotoxin IT efficacy.

G71 COMPARISON OF IMMUNOTOXINS MADE WITH RICIN, DIPHTHERIN TOXIN, CLONED DIPHTHRIA TOXIN FREQUENT AND TOXIN A CHAINS. Colombatti M,[1] Greenfield L,[2] Uckum F,[3] Vallera DA,[3] and Youle RJ[1]. [1]National Institutes of Health, [2]Cetus Corp., [3]University of Minnesota.

The cytotoxic activity of Immunotoxins (IT) made with ricin and diphtheria toxin (DT) and with two monoclonal antibodies against T cell specific antigens CD5 (mAb T101) and CD3 (mAb UCHT 1) was investigated. UCHT 1 linked to DT had exceptional properties: it killed normal human T cells and T leukemia cells (Jurkat) at 2-10 pM, a concentration 10-100 fold lower than UCHT 1 ricin and 10-500 times lower than native DT. Human multipotent stem cells were not killed up to 2000 pM UCHT 1-DT. UCHT 1-DT showed greater selectivity between T cells and stem cells than UCHT 1-ricin and may better prevent GVHD in allogeneic BMT. UCHT 1-DT killed 90% of Jurkat cells within 2 hr at concentrations non toxic to stem cells. UCHT 1-ricin required 18 hr to kill one log of target cells. UCHT 1-DT was also 100 times more potent and 5-10 times faster than T101-DT and T101-ricin. UCHT 1 was linked to DT A chain (DTA) or to a genetically engineered DT toxin (MspSA) lacking the C-terminal portion of the B chain, and their cytotoxicity compared to UCHT 1-DT. UCHT 1-MspSA was 100 fold less potent than UCHT 1-DT and UCHT 1-DTA was 100 fold less potent than UCHT 1-MspSA. NH_4Cl blocked the cytotoxic effect of DT, UCHT 1-DT and UCHT 1-MspSA but not UCHT 1-DTA. Cell surface DT receptors did not appear to be involved in the enhanced activity of UCHT 1-DT since CRM 197 blocked DT toxicity 100 fold but had no effect on UCHT 1-DT. Treatment of DT, UCHT 1-DT and UCHT 1 MspSA at pH 4.0 inactivated all three, indicating that proper toxin conformation was required for DTA entry. We conclude that the C-terminal region of DT B chain facilitates entry to the cytosol beyond initial cell surface binding.

G72 ANTI-TUMOR ACTIVITY OF IMMUNOTOXINS IN NUDE MOUSE MODEL OF HUMAN OVARIAN CANCER, D. FitzGerald*, T. Hamilton†, R. Ozols†, M.J. Bjorn+, R. Ferris+, J. Winkelhake+, A. Frankel+, M. Willingham*, I. Pastan*; *LMB, NCI, Bethesda, MD 20892; †Med. Br. DCT, NCI, Bethesda, MD 20892; +Cetus Corp. Emeryville, CA 94608

We have reported the construction of several immunotoxins that have in vitro cytotoxic activity against various ovarian cancer cell lines (Pirker et al., 1984 Cancer Res. 45:751-757) and Pirker et al, 1985 J. Clin. Invest, 76:1261-1267). The most active of these are currently being tested for in vivo anti tumor activity in a nude mouse model of human ovarian cancer. Routinely, NIH: OVCAR-3 cells (60×10^6) are inoculated into the peritoneal cavity of nude mice to produce a lethal ascites tumor--mice die between 35 and 45 days post inoculation of tumor cells. Specific inhibition of tumor growth, due to immunotoxin, is evident when there is increased survival of treated mice compared to untreated or control-treated animals. Here we report that immunotoxins made from monoclonal antibodies to the human transferrin receptor (TFR) prevent the growth of ovarian ascites tumors. Immunotoxin treatments were administered on days 5, 8 and 12 after inoculation of tumor cells and were given IP in 0.5 ml of normal saline with 10 mg/ml human albumin. The administration of 30 or 100 µg of anti-TFR (454A12) coupled to Ricin A chain prolonged survival of the mice routinely to 100 days or longer. A significant but less pronounced increase in survival was also noted when 10µg or 3µg of immunotoxin was given. Injections of antibody alone (up to 500 µg) or irrelevant immunotoxins did not increase survival. The administration of an immunotoxin made by conjugating Pseudomonas Exotoxin to a second, independently-derived, anti-TFR monoclonal antibody (HB21) was similarly active but at lower doses than the Ricin A immunotoxin.

G73 BINDING, UPTAKE AND PROCESSING OF ABRIN IMMUNOTOXINS. Godal, A., Fodstad, Ø., and A. Pihl. Institute for Cancer Research, The Norwegian Radiumhospital, Montebello, 0310 Oslo 3, NORWAY.

During a study of anti-melanoma antibodies conjugated to abrin and ricin, we obtained evidence that the mechanism of action of such immunotoxins (ITs) may be more complex than previously recognized. Thus, when different melanoma cell lines were exposed to the ITs, the sensitivity of the different cell lines to the conjugate increased with increasing sensitivity to the native toxins. When binding, uptake and intracellular degradation of a conjugate of the anti-melanoma antibody 9.2.27 and abrin were studied in melanoma lines with different sensitivities, it was concluded that the high activity of the IT in abrin-sensitive cells could not be accounted for by unspecific binding. Evidence was obtained that, after specific binding of the IT to the antigen, intracellular processing of the IT occurs along two routes; the "antigen-route" and the "abrin-route", and that the relative significance of the latter is correlated to the sensitivity of the cells to native abrin. Moreover, in the presence of lactose, when the intoxication was directed to occur exclusively along the "antigen-route", a higher rate and extent of intoxication occurred in abrin-sensitive cells, indicating that such cells have a more general high efficacy to internalize and process proteins, including the IT.

G74 **ALTERATION OF MURINE IMMUNE RESPONSE(S) BY PSEUDOMONAS AERUGINOSA EXOTOXIN-A**, Dale F. Gruber, and Thomas A. Davis, Experimental Hematology Department, Armed Forces Radiobiology Research Institute, Bethesda, MD 20814-5145.
Trauma upsets immunological balance(s) resulting in immuno-dysfunction(s) which place the host at risk. Most forms of P. aeruginosa, a commensal enteric, elaborate exotoxin. We investigated the virulence enhancing property of exotoxin by examining perturbations in mitogen induced splenic lymphoid transformation characteristics. Cells cultured in the presence of 10^{-4} to 5×10^{1} ng exotoxin over 72 hours exhibited a reproducible sensitivity to ^{3}H-TdR uptake. Overall, the order of sensitivity was CON-A $>$ PHA $>$ LPS. In general, increasing concentrations resulted in proportionately greater ^{3}H-TdR inhibition. To determine if there were temporal relationships between contact time and inhibition, cells were incubated in the presence of exotoxin (2000 ng/ml) from 1-3 hours and washed (1-3X). CON-A response levels decreased to 25 $\pm$ 7% of controls following a one hour incubation. This corresponded to levels exhibited over 72 hours in the presence of 10 ng of exotoxin. PHA respondent cells decreased to 89 $\pm$ 4% of controls, while LPS respondent cells demonstrated little, if any, inhibition. ^{3}H-TdR uptake kinetics were not significantly altered by increases in the temporal availability, nor by increasing the number of washings. Trypan blue cytotoxicity is evident at 48 hours, and later, only in populations of cells cocultured in the presence of CON-A. These data seem to imply that the CON-A mitogen respondent population was the most sensitive to the inhibitory effects of exotoxin and was dose dependent at lower levels. The inhibitory effects occurred within the first hour of contact and could not be abrogated with numerous washings.

G75 NON-SPECIFIC REACTIVITY OF MURINE MONOCLONAL ANTIBODIES (MOABs) AND MOAB-DRUG CONJUGATES WITH HUMAN AND MONKEY PMNs. David A. Johnson, Magda C. Culwell, Bennett C. Laguzza, Philip Marder, and Cynthia L. Nichols; Immunology Research, Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN, 46285.

Indirect immunofluorescence analysis by UV microscopy and flow cytometry revealed that murine MOAB-IgGs reacted with the surface of polymorphonuclear leukocytes (PMNs) isolated from normal human donors. Under identical conditions no reactivity was observed with mononuclear lymphoid cells. Ten of ten MOAB-IgGs reacted with PMNs to some degree and there appeared to be no correlation in the strength of fluorescence with the sub-isotype of the IgG. The one IgM MOAB tested, however, showed much reduced reactivity. Reduced reactivity was also observed with IgG FAB'2 fragments, suggesting that the interaction with PMNs may be Fc-receptor mediated. A similar pattern of reactivity was observed with freshly isolated Rhesus monkey PMNs.

In light of efforts in numerous laboratories to develop murine antibody-drug conjugates for use as therapeutic agents, we evaluated the interaction of such conjugates with PMNs and tested the effects of this interaction on PMN function. MOABs conjugated to 4-desacetyl vinblastine hemi-succinate were found to be reactive with human PMNs in the indirect immunofluorescence assay, though there was a slight reduction in fluorescence relative to that obtained with unmodified MOABs. Reaction of desacetyl vinblastine conjugates or free MOABs did not affect the ability of the PMNs to respond with an oxidative burst as measured in nitro-blue tetrazolium dye reduction assays. The data suggest the interaction of MOAB-drug conjugates with PMNs via Fc-receptors may have no clinically significant effect on the cells. This conclusion is supported by observations that in vivo administration to Rhesus monkeys of repeated, increasing doses of MOAB-desacetyl vinblastine conjugates resulted in no clinically significant long term effects on PMN profiles (T.F.Bumol and G.C.Todd, Personal Communication).

G76 INHIBITION OF THE IMMUNE RESPONSE TO THE ACETYLCHOLINE RECEPTOR WITH ANTIGEN-TOXIN CONJUGATES. Keith A. Krolick and Colleen A. Olsberg. Univ. Texas Hlth. Sci. Ctr., San Antonio, Texas 78284.

Myasthenia gravis is an autoimmune disease in which neuromuscular transmission is impaired, probably due to circulating antibodies directed against the acetylcholine receptor (AChR) found in the postsynaptic membrane of the neuromuscular junction. Injection of purified AChR into rats produces chronic symptoms very similar to those demonstrated by myasthenic patients. Onset of symptoms in rats with experimental autoimmune myasthenia gravis (EAMG) is highly correlated with the presence of serum titers of anti-AChR antibodies. The generation of memory lymphocytes which will make anti-AChR antibodies when challenged in tissue culture is also apparent. The goal of this study is to demonstrate in vitro the ability to selectively eliminate such AChR-reactive lymphocytes (and their subsequent immune response) by binding with AChR which has been covalently coupled to the toxic A chain of ricin. The resulting inhibition is specific in view of the lack of such inhibition of anti-KLH antibody responses. Furthermore, when AChR-immune lymph node cells were treated with AChR-A chain and then adoptively transferred into naive recipient rats, they were unable to produce serum AChR antibody or cause symptoms of EAMG. Thus, it is possible, in principle, to eliminate clones of antigen-reactive lymphocytes with antigen-ricin A chain immunotoxins. This leaves open the possibility of using such agents in immunotherapeutic approaches to autoimmune disease.

G77 INHIBITION OF PROTEIN SYNTHESIS IN MOPC 315 MYELOMA CELLS BY MONOCLONAL ANTI-IDIOTYPIC ANTIBODY-RICIN A CHAIN CONJUGATES, Alexander Marks*, Melanie Lei* and Reuben Baumal+. *Banting and Best Department of Medical Research, University of Toronto, +Department of Pathology, Hospital for Sick Children, Toronto, Canada, M5G 1L6

Previous studies of monoclonal antibody-drug (toxin) conjugates have utilized monoclonal antibodies to cell surface antigens which are not secreted. Compared to the target cells chosen for these studies, myeloma cells differ in that the immunoglobulin (Ig) they synthesize is both anchored in the membrane and secreted, with the synthesis of secreted Ig greatly exceeding that of membrane Ig. Since the idiotype of Ig on the surface of myeloma cells represents a clonally expressed tumor-specific marker, monoclonal anti-idiotypic antibodies (AIA) to myeloma Ig may provide a vehicle for specific delivery of cytoxic drugs or toxins. In order to address this question, we have examined the interaction of MOPC 315 mouse myeloma cells with AIA directed to the IgA produced by these cells. Three monoclonal AIA to MOPC 315 IgA, G3(IgG2b), A2(IgGI) and D10 (a hybrid molecule consisting of γ1 and γ2a heavy chains), were characterized with respect to their binding constants (Ka) to MOPC 315 mouse myeloma cells. The Ka of G3 and A2 was 10^8/mole; and that of D10 was $3x10^7$/mole. The AIA did not bind to a non-immunoglobulin (Ig) producing subclone of MOPC 315 cells (MOPC 315.36). Immunotoxins derived by conjugating ricin A chain (RTA) to G3 and A2 but not to D10 preferentially inhibited protein synthesis in MOPC 315 over MOPC 315.36 cells. These results suggest that the effectiveness of these immunotoxins assessed on the basis of their targeted cytotoxicity against MOPC 315 cells was dependent on the Ka but not on the Ig subclass of the AIA component of the immunotoxin.

G78 OPTIMIZING IMMUNOTOXIN ACTIVITY. Jon W. Marsh, Laboratory of Molecular Biology, NIMH, Bethesda, MD 20892

The toxicity of ricin-(anti-Thy-1.1) antibody conjugates was found to be highest when the conjugate possessed two ricin molecules per IgG. Further optimization has involved the introduction of a peptide-linker between the ricin and IgG molecule, resulting in a 10 fold increase in potency. Incubation of these conjugates with mouse leukemic cells (AKR SL2) in the presence of lactose resulted in kinetics of protein synthesis inhibition approximating that of native ricin, although not on equal molar dosages. As with ricin, the conjugate toxicity was potentiated with alkaline pH's and NH_3, and diminished by acid pH's. These findings, along with binding studies correlating ricin-membrane interaction with conjugate efficacy, have suggested that whole-ricin conjugates utilize the native toxin's route of entry.

G79 ANALYSIS OF MONOCLONAL ANTIBODY PROCESSING AFTER BINDING TO ANTIGEN ON THE TUMOR CELL SURFACE. Siegfried Matzku, Wolfgang G. Dippold, Josef Brüggen, Volker Schirrmacher, and Wolfgang Tilgen. Institute of Nuclear Medicine, German Cancer Research Center, D-6900 Heidelberg, FRG.

The nature of antigen recognized by monoclonal antibody (MAb) on the cell surface will greatly influence the fate of the immune complex, which may be envisaged as 1) stable anchorage to the surface, 2) internalization, and 3) shedding. We classified a series of anti-tumor MAbs according to these categories by analysing the kinetics of binding and release, the reversibility of binding by acid buffer treatment and the ultrastructural localization of bound MAb. In addition, paired label experiments in nude mice were performed to correlate immune complex processing with the rate of tissue accumulation. Beside the fact that we became able to predict in vivo accumulation performance of individual MAbs from in vitro data, we found out that internalization was favouring accumualtion in the tumor. This is not trivial, since in most normal cell systems, internalization is quickly followed by degradation and label excretion. The effect was very pronounced in the ESb-M tumor system, leading to highly selective accumulation of labeled MAb in tumor metastases despite the presence of antigen-positive normal cells in spleen and lymph nodes.

G80 ELIMINATION OF NEOPLASTIC B CELLS FROM HUMAN BONE MARROW. Richard D. May, Jonathan W. Uhr, and Ellen S. Vitetta. Department of Microbiology, UTHSCD, Dallas, Texas 75235

In previous studies in our laboratory, we have achieved 2 logs of in vitro killing of clonogenic Daudi cells in the presence of normal human bone marrow using ricin A chain immunotoxins (A-IT). No demonstrable damage to the CFU-E, BFU-E, and CFU-GM was found using A-IT conjugates prepared with intact immunoglobulin. Subsequently, we have employed strategies for achieving more effective specific cytotoxicity which use 1) univalent Fab or F(ab') fragments to construct the A-IT, 2) a second "piggyback" B-IT to potentiate the killing of the A-IT, and 3) agents which raise the pH of the endolysosome or alter the membrane (e.g., ammonium chloride, chloroquine, and monensin) to further enhance killing. Using these more recently developed protocols, we can kill more than 5 logs of Daudi cells. Using limiting dilution techniques, we are currently employing the above mentioned strategies to achieve 1) the maximal log killing of Daudi cells in the presence of human bone marrow cells and 2) the minimal damage to progenitor cells in the bone marrow during these treatments.

G81 ERADICATION OF TUMORS IN VIVO WITH WHOLE RICIN-MONOCLONAL ANTIBODY COMPLEXES. J. Kanellos, G.A. Pietersz and I.F.C. McKenzie. Research Centre for Cancer and Transplantation, Univeristy of Melbourne, Parkville, Victoria, 3052, Australia.

Because of the systemic toxicity of whole ricin in vivo most in vivo experiments have concentrated on the use of ricin-A-antibody conjugates. Such conjugates have little non-specific toxicity, but the toxic effect of the ricin is substantially impaired. We have prepared conjugates of whole ricin-monoclonal antibodies and used these, in vivo, for the treatment of tumors. Particular attention was paid to the conjugation protocol which rendered whole ricin non-toxic in vitro by the specific blocking of the galactose binding site of the B chain and in vivo these conjugates are not non-specifically toxic in mice. In three different models, anti-Ly-2.1 Mab-whole ricin conjugates could be shown to be effective in vivo: (a) Intraperitoneal tumors: thymomas growing i.p. could be completely eradicated by ricin-Mab; (b) Subcutaneous tumors: mice with tumors ~.5cm in diam received intravenous doses of ricin-Mab and a substantial reduction in tumor size was achieved; (c) Local treatment: ricin-Mab injected directly into tumors led to their disappearance in 48 hrs; in ~40% there was no recurrence. Thus whole ricin can be successfully used in vivo and eradicate tumors in some sites; such conjugates appear to be the most potent of all produced thus far.

G82 RECEPTOR MEDIATED ENDOCYTOSIS OF ANTI-T CELL IMMUNOTOXINS, Oliver W. Press*, Ellen S. Vitetta+, Andrew G. Farr*, and Paul J. Martin*, *University of Washington, Seattle 98104, and +University of Texas, Dallas 75235.

We have synthesized 4 anti-T cell immunotoxins (ITs) by conjugating ricin A chain to the pan T cell monoclonal antibodies 9.6 (CD2), 10.2 (CD5), 35.1 (CD2), and 64.1 (CD3). Equivalent cell surface binding to human blood T cells was demonstrated for all 4 reagents using fluorescein-conjugated goat anti-mouse immunoglobulin and rabbit anti-ricin A chain antisera. However, only 10.2-A and 64.1-A were effective at ablating the response of blood T cells to the mitogen, phytohemagglutinin (^{3}H-leucine incorporation was reduced by 99% for 64.1-A, 96% for 10.2-A, but by 0% for 9.6-A and 35.1-A using 10^{-8}M IT and 20 mM NH_4Cl). To investigate possible explanations for such differences in IT efficacy, we analyzed the process of endocytosis of ITs 10.2-A, 35.1-A, and 64.1-A by electron microscopy using a monovalent peroxidase-conjugated Fab' goat anti-mouse immunoglobulin probe. All ITs labeled T cell surface membranes in a circumferential manner at 0°C. Warming cells to 37°C resulted in rapid patching and endocytosis of ITs through both coated and uncoated membrane invaginations. Appearance of intracellular peroxidase label occurred first in small labeled vesicles (receptosomes) which fused with one another to form larger labeled vacuoles. Ineffective IT 35.1-A was delivered to lysosomes more rapidly than effective ITs 10.2-A and 64.1-A; early degradation of the anti-CD2 IT may have prevented its ricin A chain moiety from reaching its ribosomal site of action. These experiments show that internalization of an IT is not sufficient to cause cell inactivation, and that differences in intracellular routing may underlie variations in IT potency.

G83 Immunotoxins generated with Restrictocin. S. Canevari, R. Orlandi, M. Ripamonti, D. Mezzanzanica, F. Conde*, M.I. Colnaghi. Istituto Nazionale Tumori, Milano, Italy *Centro Ramon J Cajal, Madrid, Spain.

The monoclonal antibody (MoAb) MBr1, raised against human breast carcinoma was coupled to Ricin-A chain. The MBr1-A chain was, on average 200 times more efficient than the uncoupled chain in inhibiting protein synthesis. However, it was unable to kill the entire population of appropriate MCF-7 cells and exhibited slow cytotoxicity kinetics on adherent target cells. Lysosomotropic substances and ionophores were tested as potentiating agents but in both cases the cytotoxicity and kinetics remained unmodified. The same MoAb was conjugated with Restrictocin extracted from Aspergillus Restrictus. This cytotoxin is a protein synthesis inhibitor but is devoid of natural receptors on the cell membranes. Its 'in vitro' and 'in vivo' toxicity was lower than that of the Ricin-A chain. The MBr1-Restrictocin conjugate was, on average, as potent as the MBr1-A chain and 600 times more efficient than the uncoupled activated Restrictocin. A Winn-assay performed on a xenogeneic model indicated that the MBr1-Restrictocin conjugate was capable of reducing the tumor take. Of the 6 animals injected with MCF-7 cells treated with the conjugate, two did not develop tumors and in the remaining 4 the tumor mass was smaller than that observed in the 12 animals injected with MCF-7 cells treated either with the MoAb alone or with the native toxin.

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G84 AN ANTI-L3T4-RICIN A CHAIN-CONTAINING IMMUNOTOXIN KILLS CLONED HELPER T CELLS *IN VITRO*, Nancy E. Street and Ellen S. Vitetta, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

Autoimmunity, immunodeficiency and neoplasia are all examples of diseases related to disregulation of the immune system. This disregulation may be manifested by changes in the normal numbers of functional helper and/or suppressor T cells. For this reason, it would be attractive to develop strategies for eliminating specific subsets of T cells which maintain the disease state. Recent studies involving the elimination of helper cells *in vivo* with monoclonal antibodies have been promising. The major focus of this project is to use ricin A chain-containing immunotoxins (A-IT) to eliminate helper T lymphocytes in order to modulate the immune response. The *in vitro* model system utilizes an MHC-restricted KLH-specific T cell clone which expresses L3T4 and Thy-1, but not Lyt-2. The helper T cells from this clone provide help to TNP-specific B cells resulting in the secretion of specific anti-TNP antibody *in vitro*. GK1.5 is a rat monoclonal antibody reactive with a determinant on mouse helper T cells designated L3T4, which is the mouse homologue for human Leu3/T4. An A-IT prepared with GK1.5 kills the cells from the helper T clone *in vitro* very effectively (the IC_{50} is $<1.6 \times 10^{-11}$M). In contrast, the antibody alone is not toxic to the helper T cells at concentrations as high as 5×10^{-8}M. The treated cells from the T cell clone are unable to provide help to the antigen-specific B cells. Experiments are currently in progress to test the GK1.5-A immunotoxin *in vivo* using antibodies which either lack or contain an intact Fc portion. The results of the *in vitro* and *in vivo* experiments will be presented.

G85 **Anti-melanoma antibodies, directed to both antigens GD2 and GD3, indicates the significans of degenerate specificity towards antigen in antitumor activity.** Jan Thurin, Magdalena Blaszczyk, Meenhard Herlyn, Yasuhiko Kimoto, Michael Lubeck, * David Elder, ¶ Ole Hindsgaul, § Karl-Anders Karlsson, Zenon Steplewski, and Hilary Koprowski. The Wistar Institute, Philadelphia, PA 19104, * Department of Pathology, University of Pennsylvania, Philadelphia, PA 19104, ¶ Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada, § Department of Medical Biochemistry, University of Göteborg, Göteborg, Sweden.

A highly specific mouse monoclonal IgG3 antibody, detecting melanoma associated gangliosides GD2 and GD3, and it's hybridoma switch variant of IgG2a isotype were both found to be promising as candidates for immunotherapy, by showing high cytotoxicity in antibody-dependent cellular cytotoxicity and complement-mediated cytotoxicity in an ^{111}In release assay , using human effector cells and complement against 6 different melanoma cells.

G86 IMMUNOPHENOTYPE/RICIN SENSITIVITY OF LEUKEMIC T-LINEAGE MARROW PROGENITOR CELLS IN ALL AND THE THERAPEUTIC POTENTIAL OF INTACT RICIN IMMUNOTOXINS(IT) IN AUTOLOGOUS BONE MARROW TRANSPLANTATION(ABMT) FOR T-ALL/LYMPHOBLASTIC LYMPHOMA , Fatih M. Uckun, Dorothea Myers,Kazimiera G. Peczalska,Susan Azemove,John H. Kersey and Daniel A. Vallera , Departments of Therapeutic Radiology,Laboratory Medicine/Pathology and the Bone Marrow Transplantation Program , University of Minnesota , Minneapolis , MN 55455

We have studied the immunophenotype of leukemic marrow blasts in 21 T-ALL pts using a panel of MoAb which define human leukocyte differentiation antigens. Eighteen of 21 pts expressed the T-lineage marker CD2(T,p50) and the number of $CD2^+$ blasts ranged from 22-97%(median:86%) Twenty pts expressed the CD5(T,p67) determinant and 34-95%(median:77%) of blasts were $CD5^+$. Notably,all pts expressed the CD7(T,p41) antigen and 51-97%(median:91%) of blasts were $CD7^+$. Marrow blasts in 18 of 21 pts expressed all 3 determinants. Based on these marker profiles, we decided to generate IT against CD2,CD5 and CD7 for potential future use as purging reagents in clinical ABMT for T-ALL. The MoAb 35.1(anti-CD2),TIOI(anti-CD5) and G3.7(anti-CD7) were conjugated to intact ricin(R) by thioether linkage. The selective cytotoxicity of IT was evaluated by inhibition of protein synthesis in leukemic marrow blasts obtained from 13 T-ALL pts. Treatment with 35.1-R , TIOI-R and G3.7-R produced 92% , 81% and 94%(median) inhibition,respectively. The immunotherapeutic potential of the generated IT was also evaluated in a novel leukemic progenitor cell assay system. Each IT was able to selectively eliminate greater than 2 logs of clonogenic T-ALL blasts. Our results establish that the CD2,CD5 and CD7 surface antigens are present on a significant number of T-lineage progenitor cells in ALL and that these blasts are sensitive to the lethal actions of ricin.

G87 CATALYTIC TOXINS FOR THE PREPARATION OF PROTOZOAN IMMUNOTOXINS, C.L. Villemez, M.A. Russell, F. Stirpe*, J.D. Irvin#, and J.D. Robertus+, University of Wyoming, Laramie, WY, *Universita Di Bologna, Bologna, Italy; #Southwest Texas State University, San Marcos, TX; +University of Texas, Austin, TX.

Immunotoxins are of potential value for the treatment of parasitic disease for much the same reason as for neoplastic disease. We have prepared an immunotoxin for a model protozoan [BBRC 125, 25 (1984)] using diptheria toxin A chain as a toxin moiety. Diphtheria toxin A chain, however, is unsatisfactory except for demonstration purposes because it is not sufficiently cytotoxic for the protozoan, and because it cannot be applied to humans. At present, the most widely used toxin for the preparation of immunotoxins is ricin. But, we found that the protein biosynthetic system of the protozoan we are using, Acanthamoeba castellanii, is immune to ricin A chain [J. Parasit. 70, 918 (1984)], as is that of another protozoan, Tetrahymena pyriformis, reported previously [BBRC 91, 1082 (1979)]. These results suggest that ricin may not be useful for parasitic immunotoxins. We report here that the type I toxins PAP-S, PAP-L, docecandrin, saporin-6, and alpha-sarcin inhibit A. castellanii protein biosynthesis as well or better than they do mammalian protein biosynthesis. On the other hand, gelonin, momordica toxin, and the type II toxins abrin, modeccin, and volkensin, as well as ricin, are unsatisfactory as potential toxic agents for the preparation of protozoan immunotoxins. These results also have implications with regard to the comparative mechanism of action of these toxins.

Cellular Interactions and Factors in Cell-Mediated Cytotoxicity; Membrane Lesions: Liposomal Delivery Systems, Cytotoxic Drugs and Receptors

G88 NK-like Cytolytic Activity Mediated Against Herpes Virus Infected Target Cells But Not YAC-1 Target Cells By Murine Thymocytes.
E.W. Ades*, J. Tang, L.D. Butler, P. Marder and D. Delong, Medical College of Georgia, Augusta, GA.*, and Lilly Research Laboratory, Indianapolis, IN.

Natural killer (NK) cells may be the primary non-primed host defense factor in resistance to tumorgenesis and virus infection. We and others have previously demonstrated the heterogeneity of NK cells morphologically, functionally and phenotypically, including, NK cells positive and negative for asialo-GM1 (ASGM1). Here, we demonstrate that a subpopulation of murine thymocytes non-reactive with anti-ASGM1 and LyT-2$^-$/L3T4$^-$ can mediate an NK-like activity against HSV-1 infected target cells. This cytolytic activity is specific for virally-infected target cells since the uninfected target cells are not lysed. Additionally, we demonstrate that YAC-1 target cells are not susceptible to lysis, whereas cell preparations from the spleen and peripheral blood lyse both HSV-1 infected cells and YAC-1 cells. Further, we demonstrate that the thymocyte cytolytic activity we observe cannot be enhanced by addition of IL-2 in vitro in contrast to splenic NK cells that lyse HSV-1 infected cells. The relevance of immature T lymphocytes as a primary antiviral defense will be discussed.

G89 ACTIVATION OF HUMAN LARGE GRANULAR LYMPHOCYTES BY RECOMBINANT INTERLEUKIN 2, Teresa Barlozzari, Robert Numerof, James Mier, New England Medical Center, Boston, MA.

Large Granular Lymphocytes (LGL) account for the natural killer (NK) activity against a variety of malignant and virus transformed cell lines. However most human tumors are rather resistant to NK lysis and significant killing is achieved only after activation by lymphokines such as Interferon (IFN) or IL-2. Activation of LGL by rIL-2 is obtained promptly within 1-2 hr. and reaches plateau levels at 18-24 hr. The rapid response is independent of γ IFN production; the culture supernatants do not have significant amounts of γ IFN until 8 hr. of rIL-2 treatment. The rIL-2 treated LGL have an increased ability to bind previously insensitive targets such as RPMI-2650, nasopharyngeal epithelioma, and RAJI, a Burkitt's lymphoma. Cold target inhibition experiments show that resistant targets are able to compete with a sensitive one (K562) only when the effectors are rIL-2 activated, demonstrating that activated LGL express a new surface structure which enables them to bind and lyse previously insensitive target cells. This phenomenon is dependent on protein synthesis since the presence of inhibitors such as cycloheximide and actinomycin D abort the activation mechanism at concentrations that do not affect viability or baseline spontaneous killing. In conclusion, the activation of NK cells by rIL-2 involves a metabolically active process which leads to a membrane perturbation and allows a broader spectrum of binding and killing ability. Characterization of the new rIL-2 inducible proteins via monoclonal and polyclonal antibodies is currently ongoing.

G90 ANTI-TUMOR AND ANTI-MITOTIC ACTIVITY AND INHIBITION OF MITOCHONDRIAL FUNCTION BY PHENETHANOLAMINES, George B. Boder, Frank W. Beasley and Roland A. Cook, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285

Quantitative determinations of DNA related fluorescence through flow cytometry in conjunction with video time-lapse and cell staining techniques has enabled us to rapidly and precisely determine cell cycle specificity of a series of phenethanolamines. These compounds when administered subcutaneously to adenocarcinoma (AC-755) tumor bearing mice, potentiate the antitumor activity of compounds such as 5-fluoruracil (5-FU), 6-mercaptopurine and vindesine without any evidence of increased toxicity. In cell culture studies, mammalian cells are accumulated only at mitosis; sea urchin mitosis is not affected. Tubulin assembly in-vitro is not inhibited by the phenethanolamines. Processing of treated cells for tubulin indirect fluorescence revealed no disassembly of microtubules in interphase cells; however, mitotic accumulation was accompanied by apparent inhibition of chromosome movement. Examination of mitotic cells by transmission electron microscopy revealed that no microtubules were associated with the centrosomes or centromeres. Quantitation of fluorescence of treated tumor cells stained with rhodamine-123 revealed an inhibition of mitochondrial function suggesting inhibition of cell mitosis by a mechanism other than binding to tubulin. Tubulin assembly appears to be inhibited at the spindle. Since it has been proposed that calcium-calmodulin is one of the major regulatory factors involved with microtubule breakdown during chromosome movement, the effects on mitochondrial oxidation potential suggests a role for mitochondrial calcium in spindle formation.

G91 MOUSE MONOCLONAL ANTIBODIES INDUCE PHAGOCYTOSIS OF TUMOR CELLS BY HUMAN MONOCYTES, Jero Calafat, Hans Janssen and Annemarie Hekman, The Netherlands Cancer Institute, Amsterdam, The Netherlands.

Monoclonal antibody (MoAb) technology has stimulated a renewed interest in passive immunotherapy of neoplastic disease. In contrast to MoAbs used as carriers of drugs or toxins, the biological effect of unmodified antibodies depends on activation of the host's humoral or cellular effector mechanisms. Since human tumor-specific MoAbs are not likely to be available for immunocherapy in the near future, it is important to investigate the interactions between murine MoAbs and human effector cells.

This study reports the capacity of human peripheral blood monocytes to phagocytise human B-non Hodgkin's lymphoma (NHL) cells coated with mouse MoAbs. The MoAbs used recognise idiotypic determinants on the surface immunoglobulin of NHL cells. MoAbs of IgG1 and IgG2a isotype were found to be equally effective in inducing phagocytosis. Preincubation of the monocytes with heat aggregated immunoglobulin from human or mouse serum inhibited phagocytosis to the same degree, suggesting involvement of an Fc receptor on the monocytes that bind both human and mouse antibodies.

G92 KINETIC STUDIES ON THE MECHANISMS OF HUMAN NK, AK AND CTL MEDIATED CYTOTOXICITY REACTIONS, Denis M. Callewaert, Cynthia L. Sevilla and Gilbert Radcliff, Oakland University, Rochester, MI 48063

Cytotoxicity reactions mediated by human activated killer (AK) cells and cytotoxic T lymphocytes (CTL) generated in one-way mixed lymphocyte culture (MLC) were investigated using kinetic methods developed for the study of human natural killer (NK) cells. No lag period is observed for K-562 lysis by MLC-generated AK cells, and the rate of isotope release remains constant for only the first 30 to 60 minutes of the reaction. While considerable variablility was observed between experiments, calculated values for the maximal velocity of AK mediated lysis of K-562 were generally substantially greater than for NK mediated lysis under identical conditions. The rate of lytic programming for AK and NK mediated reactions were more comparable, suggesting that enhanced cytotoxicity exhibited by AK cells is due primarily to an increase in the number of competent cytotoxic cells rather than a difference in lytic function. In contrast to NK and AK mediated reactions, a lag period of 15 to 30 minutes is observed for lysis of PHA-blasts by MLC-generated human CTL, followed by a linear increase in isotope release for 2 to 4 hours. These data are interpreted in terms of a common post-binding lytic mechanism for cellular cytotoxicity reactions.

G93 Human cytotoxic T lymphocyte (CTL) clones with anti-melanoma reactivity interact with melanoma associated cellular adhesion molecules. Jan E. de Vries[1], Anje te Velde[2], Gerrit Keizer[2] and Hergen Spits[1] 1 UNICET, Immunology laboratories, 69572 DARDILLY, FRANCE and 2 the NCI, AMSTERDAM.

CTL-clones with anti-melanoma reactivity were isolated from mixed lymphocyte cultures or mixed lymphocyte tumor cell cultures, in which lymphocytes from melanoma patients were activated by allogeneic EBV-transformed B cell line cells or autologous melanoma cells. These CTL clones killed autologous and allogeneic melanoma cells preferentially, but had no NK activity as measured against K562. Blocking studies with monoclonal antibodies (moabs) showed that the reactivity of these clones was not MHC-restricted. In addition, T3, T4 or T8 were not associated with the cytotoxic reactivity. By using moabs raised against human melanoma cells that were selected on their capacity to inhibit the anti-melanoma reactivity of the CTL clones we found that the CTL clones interacted with a melanoma associated proteoglycan (M.W. 450 Kd-250 Kd) and a molecular complex consisting of 4 subunits with M.W. of 120, 95, 29 and 23 Kd. The latter antigen was found to be associated with the adhesion, spreading and motility of human melanoma cells. In addition to their membrane localization, both antigens were also found to be present in the extracellular adhesion plaques. These results indicate that the CTL clones with anti-melanoma reactivity interact with melanoma associated cellular adhesion molecules which may be involved in the dissemination of human melanoma. However the T cell receptor/T3 complex is not involved in this interaction.

G94 H-2 RESTRICTION OF REOVIRUS SPECIFIC CTL, Suezanne Emison and Duane Sears, University of California, Santa Barbara, CA 93106

The antigen recognition properties of murine cytotoxic T lymphocytes (CTL) have been characterized in terms of the restrictions imposed by individual murine H-2 (class I) antigens on the recognition and lysis of reovirus infected cells by CTL. Reovirus is a nonenveloped double-stranded RNA virus. The σ_1 outer capsid protein of the virus is a major target antigen of CTL as well as antibodies. In the present study CTL were generated against reovirus serotype 1 and 3 which differ significantly in their σ_1 proteins. The H-2 restriction pattern for reovirus specific CTL is broad with respect to the number of possible H-2 restricting elements. However, anti-reovirus CTL are highly specific; CTL restricted to one class one antigen do not effectively cross lyse infected cells expressing the inappropriate H-2 antigen. The fine specificity of this CTL response is presently being investigated at the level of H-2 antigen structure. Of interest are anti-reovirus CTL restricted to the mutant H-2D/L^{dm1} antigen, a spontaneously arising hybrid of the H-$2D^d$ and H-$2L^d$ antigens. H-2D/L^{dm1}, H-$2D^d$, or H-$2L^d$ restricted anti-reovirus CTL do not effectively cross-lyse reovirus infected cells expressing one of these other H-2 antigens. The present investigation focuses on the potential functions that individual H-2 antigen domains, and portions thereof, have in determining CTL restriction patterns.

G95 PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF LOSS-VARIANTS FROM CTL HYBRIDOMAS. Z. Eshhar[1], T. Waks[1], M. Davis[2], T. Yokota[3] and K. Arai[3]. The Weizmann Institute of Science, Rehovot, Israel[(1)], Stanford Medical School[(2)] and DNAX Research Institute, Palo Alto, U.S.A.

We took advantage of the instability of cytotoxic T cell hybridomas for the isolation and characterization of variants defective in various stages of target cell recognition, lytic process and inductive secretion of interleukines. The hybridomas specifically interacted with, and lysed EL4 target cells. Upon stimulation with either antigen or mitogen, a potentiation of the lytic activity and secretion of IL-2, IL-3 and GM-CSF was observed. Subclones of the following phenotypes have been obtained: Some lost both killing activity and inducibility, others did not kill, neither could be induced with antigen while their ability to respond to mitogen was not impaired. Few variants amongst these which lost their ability to kill could still undergo antigenic stimulation for factors secretion. None of the clones could retain cytotoxic activity without mitogen-induced IL-2 production. We suggest the coexistence of two separate receptors, one for antigen and one for mitogen. The killing activity of the hybridomas is unilaterally associated with the production of growth factors. We have cloned the α,β and γ T cell receptor genes, the IL-2 and IL-3 codding genes from cDNA library prepared from two independent parental hybridomas. Sequence analysis and restriction enzyme mapping revealed that although the hybridomas had similar antigenic and allorestricted specificity, they differ in the variable region of both the α and β chains of the TcR. Deffective subclones have been identified that do not transcribe the α,β or γ chains. Some functional clones lost their γ chains without impairing the respond to the target cells.

G96 TRIGGERING OF THE LETHAL HIT IN HUMAN CTL: DETECTION OF CYTOTOXICITY AGAINST BYSTANDER TARGET CELLS, Bernhard Fleischer, Department of Medical Microbiology & Immunology, University of Ulm, Germany.

The mechanisms by which cytotoxic T lymphocytes (CTL) kill target cells as well as the molecular signals by which the lytic machinery of the CTL is activated are not known. We have investigated the triggering of specific and nonspecific cytotoxicity of human CTL clones. Monoclonal antibodies against the T3 antigen both blocked antigen-specific cytotoxicity and triggered nonspecific cyttotoxicity against third party targets. Two different mechanisms were operating in anti-T3 induced cytotoxicity: some target cells required Fc-receptor mediated crosslinking of CTL and target, whereas with other targets bystander lysis was observed. Evidence for bystander-type of lysis was the effective triggering by $F(ab)_2$ fragments, lysis of Fc-receptor negative target cells and the resistance to inhibition by immune complexes. Bystander lysis was also triggered in CTL incubated with the calcium ionophore A23187. After extended assay times lysis of bystander targets could also be observed after specific triggering of the CTL with their specific target cell. This was again dependent on the target cell used as bystander. Targets resistant to this bystander lysis were also resistant to lysis triggered by $F(ab)_2$ fragments of anti-T3 antibodies. No soluble cytotoxic activity could be found in the supernatants of triggered CTL.

G97 RED CELL GHOST CARRIERS AT STAGES DURING THEIR FORMATION BY SEM, Cynthia L. Freehauf and Carol A. Kruse, Denver General Hospital, Surgical Oncology, Denver, CO 80204

Red cells as carriers for exogenous agents is recognized as a useful system for research and clinical applications. The properties of red blood cell ghosts (RBCGS) are dependent upon the technique used for encapsulation. RBCGS were made by two osmotic stress techniques (slow dialysis and preswell) and by electroporation. RBCGS loaded with methotrexate (0.8-1.2 mg MTX/ml RBCG) by the three techniques were examined by scanning electron microscopy at various stages during their formation to determine what factors are critical to their development. In the slow dialysis method, RBCS dialyzed against hypotonic buffer change to a homogeneous population of echinocytes. When isotonic conditions are restored, large pores and/or depressions (0.1-0.8 μm) appear in RBCGS of variable size. Upon annealing the RBCGS at 37°C, a more uniform population of RBCGS with stomatocytic and discoid morphologic forms are present; membrane perturbations and pores are still evident. In the preswell method, RBCS preswollen with 160 mOsm buffer still retain a discoid appearance. When the RBCS are lysed, almost all have an echinocytic shape and reduced mean cell volume. This morphology persists after isotonicity is restored. Upon annealing the RBCG at 37°C a stomtocytic shape returns. Very few pores are evident. For the electroporation method, RBCS appear as rounded spherocytes with small spiculations immediately after pulsing at 6 KV/cm, 16 μs. The vesiculation decreases upon incubation at 0°C for 30 min. Upon annealing at 37°C the morphology returns to stomatocytic form with no pores evident in their membranes. At higher field intensities or prolonged microsecond pulses, pores appear as seen in RBCGS from the slow dialysis method.

G98 THE EFFECT OF VESICLE SIZE ON THE IN VIVO TISSUE DISTRIBUTION OF LIPOSOME-ASSOCIATED ADRIAMYCIN

A. Gabizon, D. Goren and Y. Barenholz. Hadassah University Hospital and Hebrew University-Hadassah Medical School, Jerusalem, Israel.

We have investigated the behavior of two populations of Adriamycin (ADM) containing phospholipid vesicles with regard to various parameters. ADM-containing liposomes were prepared by ultrasonic irradiation, the lipid composition being phosphatidylglycerol, phosphatidylcholine and cholesterol (molar ratio 3:7:4 respectively). The vesicles were fractionated by preparative ultracentrifugation (150,000 g x 1 hr). Pellet (P) vesicles and supernatant (S) vesicles were then separated from unentrapped ADM by gel filtration. P and S liposomes differed in size (P, uni and oligolamellar, mean =115 ± 25 nm; S, unilamellar, mean = 35 ± 14 nm). Drug entrapment per unit of lipid was ∿3 times higher in P liposomes (P, 160 mmol ADM/mol phospholipid; S, 50 mmol ADM/mol phospholipid). Tissue distribution and toxicity studies were performed after i.v. administration of both types of vesicles into mice. The distribution and clearance of ADM in S liposomes followed a pattern different from that of ADM in P liposomes and resembling that of soluble ADM (Gabizon et al. Cancer Res. 42: 4734, 1982). In agreement with these differences in patterns of tissue distribution, preliminary toxicity studies showed that ADM in P liposomes is significantly less toxic than ADM in S liposomes. These results suggest that very small unilamellar liposomes are unfavorable carriers for an amphipathic drug such as ADM.

G99 MODEL MEMBRANES CONTAINING PURIFIED H-2K^b, CYTOSKELETAL PROTEINS, AND DEFINED LIPIDS IN A STUDY OF CYTOTOXIC T CELL-TARGET CELL INTERACTION.

Penny J. Gilmer and Steve D. Figard, Department of Chemistry, Florida State University, Tallahassee, FL 32306-3006

H-2K^b was purified by monoclonal antibody (B8-24-3)-affinity chromatography and reconstituted with defined lipids and cytoskeletal proteins into vesicles by the detergent-dialysis method. Sucrose density fractionation of reconstituted vesicles and Pronase E cleavage studies suggested that the cytoskeletal proteins aided in the incorporation and vectorial orientation of the antigens into large, cholesterol-containing membrane vesicles. Specific conjugate formation between allogeneically primed cytotoxic T cells ($\underline{H\text{-}2^d}$ anti-$\underline{H\text{-}2^b}$) and $\underline{H\text{-}2^b}$ target cells was reduced by 50% of the maximum inhibition observed by vesicles containing 6 ng purified H-2K^b plus 28 ng cytoskeletal proteins. Specificity of the response was demonstrated using purified H-2K^k proteins where only slight inhibition of conjugate formation was observed at significantly higher concentrations. Studies are underway to compare properties of vesicles formed with different membrane compositions than those used to date (0.28, 0.25, 0.47 mol fraction cholesterol, dimyristoylphosphatidylcholine, and dipalmitoylphosphatidylcholine, respectively).

G100 ROLE OF POTASSIUM CHANNELS IN KILLING BY HUMAN NATURAL KILLER CELLS. Sidney H. Golub, Lyanne C. Schlichter and Neil Sidell. UCLA School of Medicine, Los Angeles, California 90024.

We have demonstrated a voltage dependent K current in human natural killer (NK) cells using the whole cell variation of the patch-clamp technique. This K current is reduced in a dose-dependent manner by a variety of ion-channel blockers (verapamil, quinidine, 4-aminopyridine, Cd) at concentrations comparable to those that inhibit natural killing, suggesting that the K channels are necessary during the killing process. The ion channels present in the target cell do not appear to play a role since: 1) two NK-sensitive cells (K562 and U937) have different types of channels; 2)blocking the only type of channel detected in K562 cells (Na channel) does not affect killing; 3) pretreatment of target cells by quinidine and verapamil do not significantly reduce their sensitivity to killing. In contrast, pretreatment of effector cells with these drugs substantially inhibit killing. By adding EDTA or channels blockers at various times in a Ca-pulse assay system, we have determined that the blocker-sensitive phase of bound conjugates strictly corresponds with the Ca-dependent "programming" stage of killing. Thus, the data suggest that functioning K channels in NK cells are necessary during the Ca-dependent "programming-for-lysis" phase of the killing process through mediation of NKCF release, In contrast, our data do not support an active role in cytolysis by ion channels in the target cells.

G101 STUDIES ON THE LETHAL HIT MECHANISM OF NK CELLS: ACTIVATION OF NKCF SECRETION BY PROTEIN KINASE C, Scott Graves and Benjamin Bonavida, Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA.

Previous studies on the NK lytic mechanism demonstrated that following interaction of target cell with effector cell, the effector cell releases natural killer cytotoxic factors (NKCF) which can then bind to and lyse the target cell. Currently, we are interested in the signaling events leading to release NKCF. Studies on other cell systems have indicated that receptor induced transmembrane signaling leads to the metabolism of phosphatidylinositol and activation of protein kinase C (PKC) by increased cytosolic Ca^{++} and diacylglycerol. We tested the hypothesis that a similar sequence of activation events occurs in human NK by comparing Con A with the phorbol ester, 12-O-Tetradecanoylphorbol-13-acetate (TPA), and the calcium ionophores, A23187 and Ionomycin, for their ability to induce release on NKCF. Ionophores (200nM) in conjunction with TPA (20ng/ml) induced release of NKCF faster (less than 1 h) than either Con A or target cells. Only NK sensitive target cells were killed by NKCF induced by TPA and ionophores. Pretreatment of effector cells with interferon enhanced release of NKCF from effector cells stimulated with either TPA and ionophores or Con A. Activation of PKC was indicated because TPA and A23187 enhanced protein phosphorylation in LGL enriched fractions. Diacylglycerol also induced release of NKCF from PBL. Dibutyryl cAMP inhibited NKCMC but failed to inhibit TPA and ionophore induced release of NKCF. These studies suggest that NKCF is released from NK effector cells within a period of time consistent with NKCMC and that protein phosphorylation maybe involved in this event.

G102 DNA OF NONHEMATOPOIETIC TARGET CELLS IS FRAGMENTED UPON CTL-MEDIATED LYSIS, Stanislaw H. Gromkowski*, Thomas C. Brown+ and Jean-Charles Cerottini*, *Ludwig Institute for Cancer Research, and +Swiss Institute for Experimental Cancer Research, 1066 Epalinges, Switzerland.

Cytolytic T lymphocytes (CTL) play an important role in resistance to virus infections and in some forms of allograft and tumor rejection. In most in vitro studies of CTL-mediated lysis, the target cells are usually of hematopoietic origin. Obviously, in vivo, CTL must destroy a wider range of target cells. Recent studies on the role of CTL surface molecules involved in effector-target interactions suggested that the requirement for LFA-1 varied according to the origin of target cells used. It has been shown previously that DNA fragmentation takes place in hematopoietic target cells lysed by CTL (but not by Ab+C). In view of the differences mentioned above, we have investigated the fate of DNA in nonhematopoietic target cells upon CTL-mediated lysis. Three murine cell lines were used as a source of $H\text{-}2^d$ target cells (1) K^d-19, a $H\text{-}2^k$ fibroblast line transfected with K^d gene ; (2) EMT6, a BALB(c ($H\text{-}2^d$) mammary tumor-derived line, and (3) 51G, a BALB/c colon carcinoma-derived line. A K^d-specific CTL clone was used as the source of effector cells. In all cases, target cell DNA was rapidly fragmented upon CTL-mediated lysis, as seen by agarose gel analysis (ladder pattern of multiples of 200 base pair fragments) and in Triton X-100 DNA-release assay. Ab+C-mediated lysis didn't trigger DNA fragmentation in any of the target used. Thus we conclude that the events triggered in the target cells during CTL-mediated lysis are the same whether or not the target cell is of hematopoietic origin.

G103 VIRAL SURFACE ANTIGENS ACTIVATE NATURAL KILLER CELL MEMBRANE CYTOTOXICITY. Anwar A. Hakim. Loyola University Medical Center. Maywood. Illinois 60153.

Hepatitis B surface antigens (HBsAg) form a complex structure and antigenicity, with four major antigenic types "adv", ayw", "adr", and "ayr". SDS-polyacrylamide gel electrophoresis showed that HBsAg contain a major protein with Mt=22,000-25,000 (P 25) and a major glycoprotein with Mt=28,000-30,000 (GP 30). In addition, up to seven minor proteins and glycoproteins with Mt= 40,000-50,000 have been reported. A major goal of the present investigations was to elucidate the structural features of the proteins which mediate activation of the cell membrane cytotoxic activities. Peripheral blood lymphocytes (PBL) from healthy adutls with (HBs Ab-sero positive) and without (HBsAb-sero negative) circulating antibodies to HBsAg were obtained before and after vaccination with HBsAg were used as effector cells. The PLC/PRF/5 cell line developed from a primary hepatocellular carcinoma of a male with a positive serum for hepatitis B virus surface antigen was used as one of the target cells, and K-265 as the other, in a four hour ^{51}Cr-labelled release cytotoxicity assay. The first cell line produces and secretes HBsAg but no other known viral markers. While the other cell line is specific for Natural Killer(NK) cells. PBL from Ab-sero negative is not, whereas PBL from HBsAb-sero positive is statistically significant cytotoxic to both target cell lines. When treated with neuraminidase (VCN), both effector cells were increased in cytotoxicity. Incubation in media supplemented with HBsAg increased extensively the cytotoxicity of PBL from HBsAb-sero positive aduts only. The chenges in cytotoxicity is accompanied by an increased cell membrane protease activity. Cellular cytotoxicity requires a protease activation to release determinats needed for the binding of the membrane to the target cells.

G104 Lymphocyte Granule-Mediated Lysis of Erythrocytes. Dorothy Hudig, Doug Redelman, and Janine Gregg. School of Medicine, University of Nevada, Reno

Enzymes may activate the granule material of the rat large granular lymphocyte tumor cells RNK-16 for lysis of erythrocytes. We evaluated the effects of 5 variables and found:

Variable (range)	Effect of increasing the variable on: Lag time	Rate of lysis	Net final lysis
Ca^{2+} (0-16 mM)	decreased	unchanged	increased to optimum at 2-8 mM
pH (6.0-9.0)	decreased	unchanged	increased to pH 8.0
Temperature (23-41° C)	decreased	increased to 35° C	unchanged
Concentration of granules	decreased	increased	increased
Concentration of r rbc's	unchanged	increased	increased

The effects of temperature and pH on the lag time indicate that some form of activation, beyond that of collision of the cytolysin with the erythrocyte, must take place.

G105 MECHANISM OF LECTIN-DEPENDENT CELLULAR CYTOTOXICITY (LDCC): ROLE AND CHARACTERIZATION OF NON-CLASS I MHC PAPAIN-SENSITIVE TARGET CELL MOLECULES INVOLVED IN LYSIS, Jonathan D. Katz and Benjamin Bonavida, UCLA School of Medicine, Los Angeles, CA 90024.

We have previously demonstrated that in both the human and xenogenic LDCC systems the human MHC class I deficient Daudi cell line can serve equally as well as the class I positive Raji cell line as a target in LDCC. We also found that papain treatment rendered both Raji and Daudi cells unsusceptible to effector cell lysis without affecting their ability to form conjugates in the presense of Con A. This parallel abolition of post-binding lytic potential by papain of both class I negative and class I positive cells suggested the existence of non-MHC papain-sensitive structure(s) important for lysis. In this current study to further understand and identify these papain structures, we have developed an antiserum to the class I negative Daudi cells by immunization of Balb/c mice. This anti-Daudi serum has been shown to inhibit the lysis of Daudi by the xenogenic LDCC effector CTL (B6$\propto$P815). Further, the inhibition is target cell specific in that it does not inhibit the killing of P815 by $\propto$P815/CTL. Interestingly, this antiserum has also been shown to inhibit the human NK-CMC reaction of PBL against U937 (NK-sensitive) target. Thus, it seems that both CTL and NK share the same stages in the cytolytic pathway. The development of monoclonal antibodies and further characterization of this target cell-specific reagent may provide some important insight into the role of target cell surface structures, other than the MHC, in the post-binding/recognition stages of lysis in LDCC.

G106 **An anti-Thy-1 Antibody Induces Cytolytic Activity and IL-2 Secretion in Memory-Like CTL-Hybridomas.** Yael Kaufmann & Tali Ozeri, Institute of Hematology, Chaim Sheba Medical Center, Tel-Hashomer, Israel.

A possible involvement of the Thy-1 molecule in activation of cytotoxic T-lymphocytes (CTL) was investigated by testing the effect of G7, an anti Thy-1 monoclonal antibody (1), on the induction of specific killing and IL-2 secretion activities of memory-like CTL-hybridomas. We have used two monoclonal CTL-hybridomas, PMM1 and Md90$^+$, which had been derived from fusions of the AKR-thymoma BW5147 with secondary CTL generated in vivo, or in MLC culture, respectively. These hybridomas can be activated by lectins or antigen to kill target cells specifically and to secrete lymphokines. G7 mAb was found to be a potent inducer of cytolytic activity and IL-2 production, being efficient as Con A or antigen-presenting cells. Thus, following 20 hours of exposure to G7, PMM1 or Md90$^+$ proliferation was partially blocked while their IL-2 secretion and specific lytic activities were induced. However, between 10 to 100 fold G7 was required to induce secretion, suggesting the existence of different induction mechanisms for cytotoxicity and lymphokine secretion in a bifunctional CTL. In contrast to it's ability to induce cytotoxicity, G7 had no effect on the lytic phase itself. A conventional anti Thy-1 antibody did not activate these hybridomas. Taken together, the data indicate that at least the G7 epitope of Thy-1 is somehow associated with the T-cell receptor of the CTL-hybridomas. 1. Gunter, K.C., Malek, T.R. & Shevach, E.M. (1984) J. Exp. Med. 159, 716. Supported by the Israeli Academy of Sciences.

G107 CHARACTERIZATION OF HUMAN CYTOTOXIC T CELL LINES REACTIVE WITH PANCREATIC ADENOCARCINOMA CELLS. Lindsey A. Kerr, Olivera J. Finn and Richard S. Metzgar. Dept. of Microbiology & Immunology, Duke University Medical Center, Durham, NC 27710.
Since it is impractical to study an autologous in vitro response in pancreatic cancer patients, we evaluated the cellular cytotoxic responses of these patients to allogeneic pancreatic tumor cell lines. Lymph node cells from 10 patients with pancreatic carcinoma have been established in culture and maintained on IL-2 containing media up to six months. They were stimulated biweekly with one of three allogeneic pancreatic tumor cell lines (HPAF, T3M4 or CAPAN). Phenotypic analyses of long-term cultures with cytotoxic activity demonstrated that these lines were heterogenous. For example, cells in culture for 42 days from one donor, and stimulated with T3M4 cells were 98% T3+, 24% T4+, 72% T8+, 2% Leu 11+, 4% Leu 7+, 97% HLA-DR+, and 46% were reactive with an antibody to the IL-2 receptor. The ability of antigen activated IL-2 dependent lines from five donors to kill tumor targets was evaluated by ^{51}Chromium release assay using CAPAN, T3M4 and HPAF cells as specific tumor targets along with the NK target K562, a T cell line Molt 4, and a melanoma line SK-MEL-28. After one month in culture, killing of some of the pancreatic tumor cell lines was greatly enhanced with 40-80% lysis noted. Neither a cloned alloreactive cytotoxic human T cell line, nor enriched NK effector cells could effectively lyse pancreatic tumor cell targets. The data suggests that the cytotoxic activity noted with long-term IL-2 cultured cells from pancreatic tumor patients stimulated with allogeneic tumor cells is not due to NK or LAK cells, and may be due to a specific T cell recognition of target antigen.

G108 DNA DAMAGE OF TARGET CELLS BY CYTOLYTIC T-CELL GRANULES P.J. Konigsberg and Eckhard R. Podack, Department of Microbiology/Immunology, New York Medical College, Valhalla, NY 10595.

Cytolytic granules isolated from cloned lines of cytolytic T and natural killer cells cause rapid, calcium dependent lysis of target cells through the action of pore forming proteins designated Perforins(Pl)(1). Granules, in addition, contain activities that mediate degradation and release of DNA from labeled target nuclei. This DNA damaging activity is essentially identical in its properties to that described previously by Russell et al(2) using intact alloreactive CTL. DNA damage is not caused by the perforins because: 1. Isolated Pl although cytolytically active, does not damage DNA. 2. Slow DNA damage by granules can still be detected after inactivation of perforins by preincubation with Ca++ or in serum containing medium. It is suggested that DNA damage by isolated granules is mediated by a lymphotoxin-like molecule. Granules kill the lymphotoxin sensitive line L929 within 48 hours. This killing activity is partly inhibited by preincubating granules with monoclonal antibodies to lymphotoxin(provided by Genentech, Inc.).

The presence of the pore-forming perforin activity together with the DNA damaging lymphotoxin-like activity in the same organelle may suggest synergism between these cytotoxic proteins and that the pore forming activity serves as a delivery system for the DNA degrading factors.

(1) Podack, E.R., and Konigsberg, P.J., 1984, J. Exp. Med. 160, 695-710
(2) Russell, J.H, Masakowski, V.R., and Dobos, C.R., J. Immunol. 124, 1100-1105

G109 PRIMING AND TRIGGERING OF TUMORICIDAL AND SCHISTOSOMULICIDAL MACROPHAGES BY TWO SEQUENTIAL LYMPHOKINE SIGNALS: INTERFERON-GAMMA AND MACROPHAGE CYTOTOXICITY INDUCING FACTOR 2, Peter H.Krammer, Claire F.Kubelka, Werner Falk and Andreas Ruppel, Institute for Immunology and Genetics, German Cancer Research Center, Heidelberg, FRG

We found a new lymphokine, macrophage cytotoxicity inducing factor 2 (MCIF2), in the mitogen-induced supernatant of a murine T cell clone in longterm culture. MCIF2 has the following properties: 1) it elutes from a Sephadex G 100 column in three molecular weight forms (10, 34, 100 KD); 2) it is acid-labile (pH 2 to 4) and heat-sensitive (80 min at 56oC); 3) it is not constitutively secreted, coexists in the same supernatant with immune interferon (IFN-γ), and synergizes with IFN-γ for induction of tumoricidal and schistosomulicidal resident peritoneal mouse macrophages. We could uncouple this synergy by a new monoclonal anti-IFN-γ antibody and showed that IFN-γ serves as the first ("priming") and MCIF2 as the second ("triggering") signal for macrophage activation. Application of the lymphokines in the reverse order was ineffective. These data demonstrate for the first time a two-step mechanism of macrophage activation by two (physiological) lymphokine signals. On the basis of the determination of the mechanism of macrophage activation for tumor cell killing and killing of the parasite Schistosoma mansoni in vitro we showed that IFN-γ plus MCIF2 are also active in vivo. These data may help to understand the role of macrophages in cancer and infectious diseases.

G110 A METHOD TO ISOLATE SUBSET-SPECIFIC T-CELL cDNA CLONES, Byoung Kwon*, Gwan Kim*, Michael Prystowsky+, Daniel Sabath+, Frank Fitch‡ and Sherman Weissman§. *Molecular Genetics Laboratory, Guthrie Research Institute, Sayre, PA 18840. +Department of Pathology and Laboratory of Medicine, University of Pennsylvania, 36th & Hamilton Walk, Philadelphia, PA 19104. ‡Department of Pathology, The University of Chicago, Chicago, IL 60637. §Department of Human Genetics, Yale University, 333 Cedar Street, New Haven, CT 06510.

A protocol which allowed us to isolate subset-specific cDNA's exhaustively has been developed. The method was to take advantage of cloned T-lymphocytes, low background cloning vector λgt10 and differential screening in a systematic manner. cDNA libraries for con A-stimulated murine helper and cytolytic T-cell clones were prepared using λgt10 as a cloning vector. A large number of helper (L2) and cytolytic T-cell (L3) cDNA clones which were not expressed in B-lymphocyte were isolated. The cDNA clones were classified again into helper T-specific and cytolytic T-specific species. Initial characterization of some of the helper and cytolytic T-cell-specific cDNA clones will be presented.

G111 ROLE OF SOLUBLE CYTOTOXIC FACTORS IN ANTIBODY DEPENDENT CELLULAR CYTOXICITY (ADCC); INVOLVEMENT OF NK Fc-RECEPTORS; Laura T. Lebow, Susan C. Wright, G. Trinchieri* and B. Bonavida, UCLA School of Medicine, Los Angeles, CA 90024 and *The Wistar Institute, Philadelphia, PA 19104.

Our labaratory has implicated the role of cytotoxic factors (NKCF) in the lytic mechanism of Natural killer cell mediated cytotoxicity (NK-CMC). We have also shown, at the single-cell level, that NK and ADCC effector cells are overlapping. Here we investigate whether ADCC and NK cytotoxic reactions share the same lytic pathway. We examined whether the antibody coated target cell for ADCC stimulated NKCF from the effector cell by its interaction with the Fc receptor. Human periferal blood lymphocytes were stimulated with either antibody coated NK-insensitive target cells, bovine gamma globulin or NK Fc-receptor specific monoclonal antibody (B73.1). Supernatants derived from these cultures mediated cytotoxic activity against NK sensitive target cells in 20 hour ^{51}Cr release assays. The target cell specificity of the cytotoxic factors paralleled that of NKCF, in that only NK sensitive cells were lysed. In subsequent experiments we tested the NK resistant variant, U9NR. Although resistant to lysis by NKCF, U9NR was still lysed in an ADCC reaction. These results demonstrate that NKCF is released when Fc-Receptors are stimulated on NK/ADCC effector cells, however, in ADCC, we propose that antibody serves to induce lytic suseptability of NKCF resistant cells. Thus, NK and ADCC effector cells may share a common pathway in the release of NKCF but, after factor release, steps involved in lysing antibody coated targets differ from NK target lysis.

G112 IMMUNE CLEARANCE OF LIPOSOMES INHIBITED BY AN ANTI-Fc RECEPTOR ANTIBODY IN VIVO, L.D. Leserman[1,2] D. Aragnol[1], Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille cedex 9, France[1], and Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA[2].

A major problem in the use of targeted therapies is that of access to the target cells, even when these cells are accessible to the circulation. These barriers include cells of the reticulo-endothelial system, which are specialized in the removal of circulating particles. In a study designed to evaluate the potential for in vivo manipulation of the circulation and of the tissue distribution of injected liposomes, mice were passively injected with anti-DNP monoclonal antibidies of the IgG_{2a} or IgG_{2b} subclasses, or were immunized with the nitrophenyl hapten bound to a protein carrier. They were then injected intravenously with ^{125}I and carbosyfluorescein-labeled, DNP-bearing liposomes. Circulation time of the DNP-bearing liposomes was markedly reduced in both actively and passively immune mice, with increased deposition of liposomes in the liver. The increased clearance of liposomes could be abrogated by injection of a monoclonal antibody directed against the murine IgG Fc receptor (2.4G2). The results suggest that clearance of ligand-bearing reagents in the face of an immune response may be modified by specific immunologic manipulation in vivo.

G113 ACTIVATED B LYMPHOCYTES FROM MAMMARY TUMOR BEARING MICE EFFECT HIGH LEVELS OF ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY, Diana M. Lopez, Ranga R. Padmanabhan, Ronald D. Paul and Lilly Bourguignon, University of Miami School of Medicine, Miami, FL 33101

Balb/c mice bearing mammary tumors exert high levels of antibody dependent cellular cytotoxicity (ADCC). In contrast with normal Balb/c, where this reactivity is greatly reduced after depletion of macrophages, in tumor bearers such procedure results only in a modest decrease of ADCC levels. Further separation of splenocytes from tumor bearing mice on nylon wool reveals that both the nylon adherent and nylon nonadherent cells are capable of acting as ADCC effectors. Fluorescent activated cell sorting of the nylon adherent cells based on the presence of immunoglobulin on their surfaces demonstrates that a 97% pure population of B lymphocytes from tumor bearers are efficient mediators of this cytotoxicity. Pretreatment of normal mice splenocytes with the microfilament disrupting agent cytocholasin D results in loss of the cells' ability to lyse the chicken red blood cell (CRBC) targets, but does not affect the levels of cytotoxicity of any of the cells preparations derived from tumor bearing mice. Electron microscopic studies of nylon adherent SIg^+ lymphocytes from tumor bearers demonstrate cells exhibiting the typical lymphocyte morphology binding to the CRBC. The nuclear membrane of the target cells in these conjugates appears to be desintegrating while the cytoplasmic membrane is still intact. These results indicate that B cells activated by the tumorigenic process exhibit a potential to lyse antibody coated target cells that may be mechanistically different from other cytotoxicity reactions.

G114 CYTOTOXIC KETO OLEIC ACIDS -RESIDENT INFLAMMAGENS IN HUMAN LUNG. *Lynn, W.S., °Lynn, D.G., *Sachs, C. *Duke University Medical Center, Durham NC 27710 U.S.A. °University of Chicago, IL 60637, U.S.A.

Four cytotoxic keto elaidic acids,(Δ 8,9,10,11) were found at postmortem to accumulate uniquely in airways of cotton workers. These cytotoxic fatty acids were not present in control subjects (cigarette users, cystic fibrosis) or in cigarettes or cotton dust. These acids are potent inflammagens which activate inflammatory cells to move and secrete, and trigger differentiation of promyelocytes. Studies on the mechanism of these effects indicate that the fatty acids initiate profound alterations in lipid metabolism of promyelocytes. Turnover of fatty acids in phosphotidylcholine, phosphoinositides and triglycerides are increased with the result that diacylglycerols accumulate. These diglycerides, once isolated, mimic the actions of the keto elaidic acids, both on differentiation and on mature inflammatory cells. Using labelled keto elaidic acids, rates of turnover of these acids into various lipids of inflammatory cells are rapid, especially in phosphoinositides. Considerable amounts of these keto acids are also present as ester lipids in alveolar macrophages (rabbits and humans). Thus, these potent mediators of inflammtion exist preformed in some inflammatory cells.

G115 PROTEOGLYCANS IN CELL-MEDIATED CYTOTOXICITY. IDENTIFICATION, LOCALIZATION, AND EXOCYTOSIS OF A CHONDROITIN SULFATE PROTEOGLYCAN FROM HUMAN CLONED NATURAL KILLER CELLS DURING TARGET CELL LYSIS. R.P. MacDermott, R.E. Schmidt, J.P. Caulfield, A. Hein, G.T. Bartley, J. Ritz, S.F. Schlossman, K.F. Austen, R.L. Stevens. Dana-Farber Cancer Inst., Harvard Medical School, Brigham and Women's Hospital, Boston, MA, 02115, and Washington Univ., St. Louis, MO, 63110. A clone of natural killer (NK) cells (JT_B18) incorporated (^{35}S)sulfate into proteoglycan molecules, which were resistant to proteolysis. The native ^{35}S-labeled proteoglycan and its ^{35}S-labeled glycosaminoglycans migrated at M$_r$ 200,000 and 50,000 respectively. HPLC analysis revealed the disaccharides to be chondroitin sulfate A (glucuronic acid - N-acetylgalactosamine-4SO_4). X-ray energy-dispersive analysis localized the proteoglycan in the NK granules. The ^{35}S-labeled cloned NK cells incubated with K562 targets exocytosed a mean of 49% of the proteoglycans during the first 60 min, with maximal release at an effector/target cell ratio of 0.5:1. Significant proteoglycan release from JT_B18 NK cells was obtained with other sensitive target cells (REX, Molt4, and CEM), but not with cells which were resistant to killing (KG1 and Laz156). In cytotoxicity blocking studies with anti-LFA-1 or with anti-TNK_{TAR} to inhibit cytolysis by NKTa+ (Ti) NK clones at the target cell level, a concomitant inhibition of proteoglycan release from NK effector cells was observed. Thus, protease-resistant intracellular proteoglycans with chondroitin sulfate A side chains are specifically exocytosed from the granules of human NK effector cells upon contact with sensitive targets, suggesting that these proteoglycans may be involved in the mechanism of cytotoxicity.

G116 SIMILARITIES BETWEEN CTL AND B CELL LFA-1-DEPENDENT ADHESIONS
Eric Martz, Department of Microbiology, University of Massachusetts, Amherst MA 01003.

LFA-1 appears to be a crucial adhesion molecule in CTL-mediated killing. We have previously characterized the initial CTL-target adhesion step in a murine system. In the present study, we have developed a new assay to characterize the spontaneous homotypic adhesions of cultured Epstein-Barr transformed human B lymphocytes. Both adhesions are inhibited by anti-LFA-1 antibodies, require Mg^{++}, are not supported by Ca^{++}, require metabolic energy, are temperature sensitive, and are inhibited by cytochalasin B. Others have reported that the adhesions of rat lymphocytes to high endothelium are Ca^{++}-dependent, and inhibited by mannose-6-phosphate and fucoidan. The latter agents do not inhibit B cell adhesions. Hence CTL-target and B-B cell adhesions are similar, and different from T-endothelium adhesions. Since the cultured B cells are easier than CTL to prepare in large quantity, and their adhesions are easier to assay, we plan to use this system to investigate the molecular mechanism of the LFA-1-dependent adhesion involved in T cell interactions including CTL-mediated killing.

G117 DNAase ACTIVITY IN CYTOPLASMIC GRANULES OF CYTOTOXIC LYMPHOCYTES (LGL AND CTL).
W.E. Munger, *C.W. Reynolds, and P.A. Henkart. Immunology Branch, NCI, Bethesda, MD 20892 and *Biological Response Modifiers Program, NCI, Frederick, MD 21701.
Target cells lysed by large granular lymphocytes (LGL) and cytolytic T lymphocytes (CTL) undergo rapid nuclear DNA breakdown. To see if granule exocytosis of a DNAase as well as the pore-forming cytolysin could account for this, we have examined purified cytoplasmic granules from various lymphoid cells for DNAase activity. Measured by ^{125}I-UdR DNA release from isolated nuclei under physiologic conditions, rat LGL and murine CTL granules contain high levels of DNAase activity; in contrast, most non-cytotoxic lymphoid cells contain only low levels of granule-associated nuclease activity. Only killer cell granules (which contain both cytolysin and DNAase) mediate both cell lysis and DNA release when tested against whole target cells. The released DNA displays extensive fragmentation when electrophoresed on agarose gels, confirming the presence of DNAase activity. Granule-mediated DNA breakdown is inhibited by zinc as reported for killer lymphocyte-mediated DNA breakdown in target cells. The granule enzyme is separable from the cytolysin by column chromatography. In addition to the above DNAase which mediates DNA breakdown in isolated nuclei at neutral pH, granule extracts also degrade purified DNA via a lysosomal, low pH optimum DNAase activity. Overall, these results support a granule exocytosis model in which killer lymphocytes in close contact with targets attack them by secreting the contents of cytoplasmic granules. One granule component, cytolysin, forms a lytic pore through which the DNAase(s) pass, creating additional damage to the target cell nucleus.

G118 COMPARISON OF MECHANISM OF CYTOLYSIS OF COMPLEMENT, CYTOLYSIN AND CYTOXINS ON CELLS BY DIGITAL VIDEO MICROSCOPY. Mary E. Neville*, Stan Zietz*+ and Jeri Lawing*. E. I. du Pont de Nemours, Central Research and Development, Glenolden, PA 19036* and Drexel University, Dept. of Mathematics and Computer Science, Philadelphia, PA 19104+.

Our investigations using digital video microscopy (DVM) suggest that one of the earliest events in lymphocyte-mediated cytolysis is disruption of nuclear/chromatin (N/Ch) organization. This effect may be caused by soluble proteins, cytoxins, which we have shown to kill NK-sensitive cells. Two other soluble cytolytic factors are complement and cytolysin[1]. Both molecules are thought to kill cells by creating pores in their plasma membranes causing death by osmotic shock. Cytolysis of cytoxins was compared to cytolysis of cytolysins and complement using DVM. Cytoxins caused changes in N/Ch organization within minutes after addition and after 4 hrs caused disruption of N/Ch structure without destruction of the plasma membrane as measured by trypan blue and napthol yellow-S staining. In constrast, cytolysis and complement caused disruption of the plasma membrane with some swelling of the nuclei without N/Ch disruption. These results suggest that cytoxins may kill in a manner analogous to cytotoxic lymphocytes by disrupting N/Ch structures while cytolysin kill by a mechanism similar to complement.

[1]P.J. Millard, M.P. Henkart, G.W. Reynolds and P.A. Henkart. J. Immunol. 132:3197.

G119 THE ROLE OF N-LINKED GLYCOPROTEINS IN NK CELL MEDIATED CYTOLYSIS. T.N. Oeltmann and W.H. Chambers. Vanderbilt University, Nashville, TN 37232

The effects of inhibitors of N-linked glycoprotein processing were examined in cells involved in natural cell-mediated cytotoxicity (NCMC). Pretreatment of non-adherent peripheral blood mononuclear leukocytes (PBL) with tunicamycin for 18 hours was shown to inhibit lytic activity against K-562 target cells. Concentrations of the inhibitor that were effective in inhibiting NCMC (2.5 - 5.0 g/ml) also altered glycoprotein processing as measured by incorporation of 2[^{3}H]-mannose. However, protein synthesis in these cells was also inhibited by as much as 50% as measured by incorporation of [^{14}C]-labeled amino acids. Castanospermine inhibited NCMC at 0.1 and 0.01 g/ml after 18 hours of pretreatment. Processing of mannose-containing oligosaccharides was altered in castanospermine treated PBL; however, protein synthesis was also inhibited by 30%. Pretreatment of PBL with swainsonine (18 hours) at 0.1 and 0/01 g/ml inhibited lysis of K-562 target cells. Processing of mannose containing oligosaccharides was altered in swainsonine treated PBL; however protein synthesis was not inhibited. Pretreatment of PBL with castanospermine or swainsonine (18 hours) had no effect on target cell : effector cell conjugate formation. Pretreatment of K-562 target cells with castanospermine or swainsonine did not change their susceptibility to lysis by PBL. These results suggest that N-linked glycoprotein(s) are involved in NCMC at the effector cell level in at least one post-conjugational step.

G120 MECHANISM OF CYTOTOXICITY BY MOUSE NATURAL CYTOTOXIC NC CELLS: MEDIATION BY TUMOR NECROSIS FACTOR (TNF). John R. Ortaldo, Llewellyn Mason, Bonnie J. Mathieson, Shu-Mei Liang*, David A. Flick† and Ronald B. Herberman, BTB, BRMP, DCT, NCI-FCRF, Frederick, MD 21701, *Biogen S.A., Geneva, Switzerland and †University of Florida College of Medicine, Dept. Immunology and Medical Microbiology, Gainesville, FL 32610

Natural cell mediated activity in the mouse has been associated with two functionally and phenotypically different types of effector cells, the natural killer (NK) cell and the natural cytotoxic (NC) cell. The prototype target cell for the detection of mouse NK activity is the YAC-1 lymphoma cell line, whereas the WEHI-164 cells are used as the target to selectively measure NC activity. During studies analyzing the mechanism of cytotoxic molecules involved in NK cytotoxicity, it became evident that WEHI-164 cells, the prototype target for NC cells, were highly susceptible to the direct lysis by human and mouse recombinant TNF. Further studies have shown that NC but not NK activities mediated by normal mouse splenocytes were completely abrogated by inclusion of rabbit antibodies to either recombinant or natural TNF. This inhibition was not seen by normal rabbit serum. Thus the activity defined as NC appears to be due to TNF release by a subset of normal spleen cells. These results provide new information regarding the mechanism of cytotoxicity of the mouse NC cell. These data, as well as previous reports, provide evidence that the TNF release from cells, presumably in the macrophage/monocyte lineage, accounts for the NC activity observed in murine spleen and bone marrow cells.

G121 IN VIVO OR IN VITRO SELECTION FOR RESISTANCE TO NATURAL CYTOTOXIC CELL LYSIS SELECTS FOR VARIANTS WITH INCREASED TUMORIGENICITY, Paul Q. Patek, Ying Lin, John Leslie Collins and Melvin Cohn, The Salk Institute, P.O. Box 85800, San Diego, CA 92103

Experiments were designed to test the hypothesis that transformed cells which are NC-sensitive must escape NC activity if they are to grow as tumors in normal individuals. NC-resistant variants were selected, either in vivo or in vitro, from NC-sensitive cell lines which grow as tumors in immune deficient mice but not in syngeneic normal mice. The tumorigenicity of cloned NC-resistant variants was compared to the parental cell lines and to cell lines which went through the selection procedure but, after cloning, remained NC-sensitive.

Cloned NC-resistant cell lines, derived from tumors that developed in x-irradiated nude mice following the injection of an NC-sensitive cell line, are tumorigenic in normal mice while cloned NC-sensitive cell lines, derived from the same tumors, are unable to grow as tumors in normal mice. Similarly, six of seven NC-resistant cloned cell lines, independently isolated after in vitro selection for NC-resistance, are tumorigenic in normal mice, while cloned NC-sensitive cell lines isolated from the same in vitro selected populations are not tumorigenic in normal mice.

Thus, either the in vivo or in vitro selection of NC-resistant cells selects for cells tumorigenic in normal mice; these findings, along with our previous observations that selection for cells tumorigenic in normal mice selects for NC-resistance, provides compelling evidence that escape from NC activity is required before some transformed cells can grow as tumors in normal mice.

G122 INTRACELLULAR REOVIRUSES SURVIVE CTL-MEDIATED LYSIS OF THEIR HOST CELL Donna M. Peters, Eric Martz, University of Massachusetts, Amherst, MA. 01002.

CTL induce rapid, extensive internal disintegration in target cells and this is unique among immune lytic mechanisms studied. This raises a question as to whether CTL are uniquely capable of halting virus infections by inducing internal damage resulting in inactivation of intracellular virus. Mouse P815 cells were infected with reovirus. The virus infection kills the host cells at about 2 days. The infectious activity (measured by PFU) was compared between host cells killed by CTL vs sonication at various points in the infection cycle of the host cell. Results indicate that CTL are incapable of inactivating intracellular reovirus at any point in the life cycle of the virus within the host.

G123 STUDIES ON THE MECHANISM(S) OF DECREASED NK ACTIVITY IN THE SPLEENS OF MAMMARY TUMOR BEARING MICE, Lourdes M. Rivera, Ronald D. Paul and Diana M. Lopez, University of Miami School of Medicine, Miami, FL 33101

Mice bearing a chemically induced mammary adenocarcinoma exhibit a significant decrease in splenic natural killer cell (NK) activity as measured by 51Cr release assay. This decrease is evident as early as two weeks post inoculation of the tumor and persists until the death of the animal 5-6 weeks later. Since we have previously described an enhancement of antibody dependent cellular cytotoxicity (ADCC) in the nylon nonadherent cells of tumor bearing mice we explored the possibility that the diminished NK activity may be related to the concurrent increase in ADCC. Large granular lymphocyte (LGL) enriched fractions from discontinuous Percoll gradients using nylon nonadherent cells from normal mice exhibited enhanced levels of ADCC. The LGL containing preparations of tumor bearers, however, did not have such an increased cytotoxic activity but rather, appeared to be residing in the fractions containing small lymphocytes. The ability of LGLs from spleens of tumor bearing mice to bind Yac-1 targets was analyzed. Although by morphological enumeration fewer LGLs were found in the corresponding Percoll fractions of the tumor bearing mice, a higher percentage of these cells were capable of binding the Yac-1 targets as compared to the LGLs from their normal counterparts. This suggests an impairment in the ability of these cells to deliver the lethal hit to their targets and/or a decrease in their recycling capacity.

G124 NATURAL KILLER (NK)-RESISTANT TUMOR CELLS BECOME SUSCEPTIBLE TO NK LYSIS UPON FUSION WITH RECONSTITUTED VESCICLES CONTAINING NK-SENSITIVE MEMBRANE STRUCTURES. Robert C. Roozemond[1], Peter van der Geer[1], and Benjamin Bonavida[2]. [1]Labaratory of Histology and Cell Biology, University of Amsterdam, The Netherlands, and [2]UCLA School of Medicine, Los Angeles, CA 90024.

Natural killer (NK) cells have been shown to be selective in their ability to lyse NK-sensitive target cells. Target cell resistance to NK cells may be due to one or more possibilities such as lack of recognition structures, inability to program the NK cell, inherent resistance to lysis, etc. To elucidate whether sensitivity to lysis is a property of the membrane constitution of the NK-sensitive target, we investigated whether NK-resistant targets become sensitive following fusion with reconstituted vesicles containing membrane components from the NK-sensitive target cell, sendai virus envelope glycoproteins, and exogenous lipids. The results of such experiments demonstrate that NK-resistant targets (both human and murine) become susceptible to NK CMC in both short-term (3h) and long-term (18h) ^{51}Cr release assays. The cytotoxic effect observed was found to depend on the concentration of reconstituted vesicles used in the fusion step. No effect was found when target cells were fused with vesicles which contained membrane constituents derived from NK-resistant targets or NK-sensitive targets from another species. These results demonstrate that NK resistance of certain targets is a property of the membrane composition. The fusion approach used, therefore, will allow detection and characterization of specific target membrane components involved in lysis.

G125 MICROSPHERES CAN FOCUS THE CYTOTOXIC ACTION OF ANTHRACYCLINE ANTIBIOTICS TO THE PLASMA MEMBRANE. Kevin L. Ross, Jean-Claude Jardillier and Zoltàn A. Tökés. Department of Biochemistry, Comprehensive Cancer Center, University of Southern California, Los Angeles, CA 90033 and Institute Jean-Godinot, University of Reims, Reims, France.

Anthracycline antibiotics such as the chemotherapeutic agent Adriamycin, Adr, are known to intercalate into double stranded DNA, inhibit enzymes involved in DNA and RNA synthesis, perturb subcellular organelles and alter membrane functions. Covalent attachment of anthracyclines to a solid support such as polyglutaraldehyde microspheres, PGL, restricts the drug interaction to the cell surface. These drug-microsphere complexes, Adr-PGL, are cytotoxic to human and murine tumor cells. Fluorescent microscopy and transmission electronmicroscopy establish that Adr-PGL has a high affinity for the cell surface, cause extensive bleb formation and show no evidence of internalization. Nuclear fluorescence occurs only with free Adr and cannot be detected with Adr-PGL. The drug-polymer complexes are effective in killing cells with acquired resistance to free Adr. An inactive anthracycline analog becomes cytotoxic when coupled to microspheres demonstrating that a new mechanism of drug action is created. Chromium-51 release studies, indicative of cell membrane integrity, have shown that Adr-PGL can cause damage more rapidly than equal doses of free Adr. Long term exposure to free Adr results in the gradual acquiring of resistance. However, this does not occur with similar exposure to Adr-PGL, demonstrating that cells cannot readily modify membrane functions leading to resistance. The experiments establish that anthracycline drug action can be restricted to the cell surface, providing a novel mechanism of cytotoxicity for which the cells cannot develop resistance.

G126 PURIFIED LYMPHOTOXIN (LT) FROM CLASS I RESTRICTED CLTs AND CLASS II RESTRICTED CYTOLYTIC HELPERS INDUCES TARGET CELL DNA FRAGMENTATION. N.H. Ruddle, K. McGrath T. James , and D.S. Schmid. Yale University School of Medicine, New Haven, CT

TNP-specific cytotoxic (Lyt 2 ,class I restricted) T cells secrete lymphotoxin (LT) under antigen or mitogen stimulation. LT from these cells kills targets in 4 hours if rapidly introduced into the cytoplasm using an osmotic technique. These CTLs initiate digestion of target cell DNA into discretely sized fragments. Lyt 1 , L3T4 ovalbumin specific helper clones secrete LT after treatment with mitogen or antigen and class II matched spleen cells. These cells also cause rapid 51 Cr release and DNA fragmentation in a class II restricted fashion if OVA is presented by the target. All these clones stain positively with antigranule antibody (Podack) after stimulation. Purified LT derived from activated T cell clones induces target cell DNA fragmentation in a similar pattern over an extended time course (24 hours). Recombinant derived tumor necrosis factor (TNF) also induces target DNA fragmentation. Interferon (IFN) does not. Combinations of lymphokines (LT and IFN) (TNF and IFN) mediate DNA fragmentation to the same degree as LT or TNF alone. The data suggest that cytotoxic and helper T cells with cytolytic activity use LT as a killing substance. Differences in time course and target cell specificity between cell mediated and lymphotoxin killing may reflect differences in the mode of toxin delivery.

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G127 SIGNALLING INVOLVED IN STIMULATION OF INTERLEUKIN SYNTHESIS AND IN INDUCTION OF CYTOLYTIC ACTIVITY IN A CYTOLYTIC T CELL CLONE (CTL), Anne-Marie Schmitt-Verhulst, Claude Boyer, Annick Guimezanes, Christine Hua, Francoise Albert and Michael Buferne, Centre d'Immunologie, INSERM-CHRS de Marseille-Luminy, FRANCE.

The activation of a mouse alloreactive H-2K^b-specific CTL clone, as measured by γ interferon (γ IFN) secretion or by lysis of alloantigen expressing or irrelevant target cells, was studied by stimulation with the alloantigen or an anti-clonotypic (anti-Ti) monoclonal antibody (mAb) in different forms, either cell-free, or cell-associated. Conditions that stimulated the CTL for production of γ IFN included: T cell receptor triggering with cell associated H-2K^b or with soluble IgG2a anti-Ti mAb or its F(ab')2 fragment, but not its Fab fragment. The phorbol ester PMA, substrate of Protein Kinase C, acted as a cofactor increasing the efficiency of cell-associated H-2K^b, or anti-Ti mAb mediated activation. Ti-mediated activation was bypassed by the synergistic action of a Ca^{++} ionophore and PMA, but not with either component alone. Specific CTL activity was obtained when the CTL interacted with H-2K^b expressing cells or with the hybridoma cells expressing surface immunoglobulin specific for the Ti, but not when it interacted with hybridoma cells expressing surface immunoglobulin specific for H-2 products of the CTL. Activation of non-specific CTL lytic activity toward the YAC target cell was obtained in the presence of the IgG2a anti-Ti mAb, but not with the F(ab')2, or the Fab fragment. PMA alone induced non-specific CTL activity, but did not act as a cofactor for anti-Ti mediated activation. The results suggest that the T cell receptor (and/or asociated structures) is involved in signalling for both activation leading to cytolysis and stimulation of interleukin synthesis, but that the intracellular activation pathways may be distinct.

G128 THE RECEPTORS INVOLVED IN NON-SPECIFIC KILLING BY CULTURED CYTOTOXIC T LYMPHOCYTES. Ken Shortman & Anne Wilson, The Walter & Eliza Hall Institute of Medical Research, Melbourne, Australia.

Although specificity for both foreign antigen and self H-2 is a hallmark of cytotoxic T lymphocytes (CTL), there are now many examples where cultured CTL kill a wide range of target cells in a non-specific manner. We have studied the non-specific killing by mouse CTL in limit-dilution culture. Virtually all Ly 2$^+$ T cells produce CTL-clones which are initially specific, but by day 8-9 are non-specific. The cells in these clones are Ly 2$^+$ L3T4$^-$, have normal DNA content and chromosome complement, and have the morphology of large granular lymphocytes. Only the larger cells within the clone carry out non-specific killing. This killing is independent of target-cell H-2 and is not inhibited by anti-Ly 2. Cold-target inhibition experiments suggested two different "receptors" were responsible for this effect, both different from the H-2 restricted antigen-specific receptor. One showed preference for YAC-1 target cells and was similar to the NK-receptor. The other showed preference for P815 target cells. Analysis of different mouse strains showed different propensities to develop these "broad range" receptors in culture; some strains developed both, some one, some the other, some neither. The mouse strains with high initial NK activity also developed the NK-like receptor on cultured CTL. The nature of these "receptors" and the factors inducing their expression on CTL are currently being investigated.

G129 INNOCENT BYSTANDER CELLS DESTROYED IN VIVO AT THE SITE OF INNOCULATED CLONED CYTOTOXIC T LYMPHOCYTES, Mary Ellen Snider and David Steinmuller, Transplantation Society of Michigan, Ann Arbor, MI 48104

Cloned cytotoxic T lymphocytes (CTL) that recognize Epa-1, a non-H-2 histocompatibility antigen expressed on epidermal cells (EC), induce necrotizing skin lesions upon innoculation into allogeneic hosts, but no reaction occurs in syngeneic hosts. However, premixing the CTL with Epa-1$^+$ EC prior to their innoculation into syngeneic hosts results in grossly observable skin ulceration. Necrosis of the antigenically innocent skin occurs only when the CTL are premixed with the Epa-1$^+$ EC since mixtures of the CTL with Epa-1$^-$ EC do not produce lesions, suggesting that specific antigen recognition is required for the non-specific tissue destruction. Evidence for this bystander phenomenon has not been obtained in vitro. For example, in cold target inhibition assays, the addition of increasing numbers of unlabeled Epa-1$^+$ EC to cocultures of CTL and labeled Epa-1$^-$ EC does not increase the release of Cr^{51} above the backround for the latter cells; thus premixing CTL with Epa-1$^+$ EC does not cause non-specific lysis of Epa-1$^-$ EC in vitro. In addition, unfractionated supernatants from the coculture of cloned CTL and Epa-1$^+$ EC do not induce tissue destruction when injected into syngeneic host skin. These data suggest that factors released from mixtures of CTL and antigen-specific target cells may not be directly responsible for the destruction of innocent bystander cells, but that as a consequence of this antigen-specific interaction, proximal cells or cells recruited to the area might be stimulated to synthesize and secrete cytolytic factors.

G130 Conjugate transformation between allospecific cytotoxic T-lymphocyte clones and target cells precedes T cell receptor antigen interaction. Hergen SPITS and Jan E. de VRIES, UNICET, Immunology laboratories, 69572 DARDILLY, FRANCE.
A single cell assay in agarose was used to study the molecular requirements of the interaction between allospecific cytotoxic T-lymphocyte (CTL) clones and target cells. This assay allows discrimination of the adhesion and the lytic phases in the cytotoxic process. Antigen specific T4+ and T8+ CTL clones were found to form conjugates with target cells which did not express the appropriate antigen indicating that T cell receptor (Ti)/antigen interaction is not required for conjugate formation.
The percentage of conjugates between the HLA-A2 specific CTL clone JR-2-16 (T8+) and human M1 fibroblasts and M1 cells transfected with -DNA coding for HLA-A2 (M1/A2 cells) was similar, but only the M1/A2 cells were lysed. Mouse L cells transfected with HLA-A2 were not lysed, since JR-2-16 did not form conjugates with L/A2 cells (or L cells). Adhesion of JR-2-16 to M1 and M1/A2 cells was inhibited by anti-LFA-1. Antibodies against class I-HLA, HLA-A2 and T8 blocked both adhesion and lysis, whereas anti-T3 moabs only affected the lytic step. Finally moabs against a public idiotype on the T cell receptor of a series of CTL clones did not inhibit conjugate formation significantly, but the lytic phase was strongly inhibited. These data indicate that 1) the interaction between Ti and its antigen occurrs after the formation of antigen non specific conjugates 2) LFA-1, T8 and HLA-antigens are associated with this antigen non-specific conjugate formation 3) antigen non-specific conjugate formation is required for T cell receptor antigen interaction.

G131 PROTONIC ACTIVATION OF CYTOTOXICITY BY ACTOMYOSIN ATPASE. Reuven Tirosh - Physics Dept., Bar Ilan University, Ramat Gan, 52100 Israel.

Vectorial flux of energetic protons generated by ATP hydrolysis along oriented actin filaments has been proposed to drive cytoplasmic streaming, cell motility and muscle contraction. Orthogonal actin filaments, homogenously attached to the plasma membrane, could generate inwardly a subthreshold flux of protons. Antigen- or cell-mediated clustering of receptors associated with such filaments can locally amplify the protonic influx: this may trigger early events of cellular activation such as PH fluctuations, depolarization and Ca^{2+} mobilization, that regulate other energetic processes like endocytosis, exocytosis, cell motility and intercellular conjugation. Various aspects of cytotoxicity mediated by Ab+C' or by cells (CTL or NK), are integrated to reveal such an early excitation leading to exhaustive activation of the target(TC). In this approach, two kinds of effector operation are expected, that have opposite pre-phagocytic-like profiles of their conjugates: In one case the stimulative flux of protons is directly induced into the TC that engulfs the effector. But in the other case, the TC is engulfed by the effector which is first stimulated by protonic influx to release cytotoxic factors. Both cases are indeed realized in reports of microscopical, physiological and biochemical observations on the cytolytic interactions of CTL and NK cells respectively.

G132 ANTIGENIC HOMOLOGY OF LYTIC PROTEINS INVOLVED IN COMPLEMENT- AND CYTOTOXIC T-CELL-MEDIATED CYTOLYSIS.

Jürg Tschopp and Danièle Masson, University of Lausanne, CH-1066 Epalinges, Switzerland.

The lytic activity of granules from cytotoxic T-cells resides on a 66 kDa protein called perforin (Masson and Tschopp, J. Biol. Chem. (1985) 260: 9096). Perforin shares many functional properties with the complement component C9. Both proteins occur as monomeric precursor which can polymerize and insert into lipid bilayer forming transmembrane lesions. In order to search for structural homology, two peptides corresponding to amino acids 74-86 and 101-111 of C9 were synthesized. This segment is rich in cysteine and is highly conserved, since it is present, besides in C9, as a repeat unit in the LDL receptor. Antibodies raised against these two peptides reacted specifically with C9, since immunostaining was inhibited by the corresponding peptides. Moreover, the antibodies bound on Western blots to complement components C6, C7, C8α-γ and to purified perforin indicating that these proteins form a new protein superfamily. The peptide corresponding to residues 101-111 of C9 inhibited at a concentration of 100 μM granule-perforin- and CTL-mediated cytolysis. These results suggest that perforin plays a crucial role in CTL-cytotoxicity and that the cysteine-rich domain shared by perforin and C9 is functionally important.

G133 ANTIGENIC CHARACTERIZATION OF CYTOTOXIC LYMPHOKINES PRODUCED BY CLONED HUMAN T CELL LINES. Carl F. Ware, Lora M. Green, Patricia Egan-Devlin and Jim Devlin. Division of Biomedical Sciences, University of California, Riverside, CA 92521 and Biogen Research Corp., Cambridge, MA 02142

Cloned lines of human T cells, derived from allogeneic mixed lymphocyte cultures or stimulation with tetanus toxoid, were characterized for their ability to mediate direct target cell lysis, cell surface phenotype and their capacity to release cytotoxic lymphokines in response to mitogenic lectins. Soluble and alloantigen specific and natural killer-like cytotoxic T cells (CTL) expressing either the $CD3^+$, $CD4^+$ or $CD3^+$, $CD8^+$ phenotypes were capable of releasing cytotoxins in response to ConA or PHA. Toxin activity was measured on the L929 cell line using the MTT colorimetric assay. Kinetic analysis of the CTL-toxin, in comparison to α lymphotoxin (α-LT) and tumor necrosis factor (TNF), indicated all three activities required 8-10 hr to lyse 50% of the L929 target cells. Specific polyclonal antiserum to α-LT failed to neutralize the cytotoxin(s) produced by a majority of CTL clones tested. Monospecific anti-TNF exhibited a partial ability (10-50%) to neutralize CTL produced toxins from selected T cell clones. However, most of the CTL clones produced a cytolytic activity that was antigenically distinct from both α-LT and TNF. These results identify a novel member(s) of the family of cytotoxic lymphokines. Supported by a grant from the National Cancer Institute, NIH CA 35638.

G134 TUMOR INDUCED SUPPRESSOR MACROPHAGE WITH INCREASED ADCC FUNCTION, Gordon A. Watson, Ronald D. Paul, and Diana M. Lopez, Univ. of Miami Sch. of Med., Miami, FL. 33101

An atypical splenic macrophage subpopulation, whose presence is associated with progression of a murine mammary adenocarcinoma, demonstrates markedly enhanced antibody dependent cellular cytotoxicity (ADCC). These morphologically distinct cells begin to populate the spleen approximately two weeks post-tumor inoculation. By five weeks, they comprise up to 30% of total cells in the spleen. After isolation by Percoll density gradient centrifugation, or flow. cytometry cell sorting, this macrophage subset was found to express Ly-5, Mac-1, Mac-2 and variable amounts of surface Ia. This is in contrast with normal splenic macrophages which comprise only 3-5% of total spleen cells and do not express Mac-2, a marker of macrophage differentiation. These Mac-1,2 positive cells effect a titratable suppression of normal blastogenic response to PHA, Con A, LPS and, more importantly, to a tumor associated antigen (TAA) preparation. These suppressor macrophages exhibit levels of ADCC comparable to those obtained with BCG activated peritoneal macrophages (74.2% vs. 79.1%) and significantly higher than normal splenic macrophages (37.6%). That the ADCC was Fc-receptor mediated was assessed by both deletion of specific antisera and inclusion of blocking antibody. The relevance of this high ADCC potential with respect to cytotoxicity within this murine tumor system will be further addressed.

G135 EVIDENCE FOR THE INVOLVEMENT OF HUMAN NKCF BUT NOT TNF IN THE NK LYTIC MECHANISM: NK-RESISTANT VARIANTS CAN BE GENERATED BY THE PROLONGED CULTURE OF U937 CELLS IN NKCF BUT NOT TNF, Susan C. Wright and Benjamin Bonavida, UCLA School of Medicine, Los Angeles, CA 90024.

In order to examine the possible involvement of cytotoxic lymphokines in the NK lytic mechanism, we have generated lymphokine-resistant variants from the NK-sensitive U937 cell line. Parental U937 cells are sensitive to lysis by natural killer cytotoxic factors (NKCF) as well as tumor necrosis factor (TNF). However, variants selected by growth in the presence of either factor are resistant to lysis by either factor. Variants resistant to NKCF (U9NR) are also resistant to lysis by NK cells in a CMC reaction. In contrast, variants resistant to TNF (U9TR) are as susceptible to NK cells as parental U937. Both variants are lysed in an ADCC reaction, whereas U9NR but not U9TR is resistant to LDCC (lectin-dependent cell-mediated cytotoxicity). The NK resistance of U937 is apparently due to a deficiency of NKCF binding sites since this line cannot absorb NKCF, although it can still form conjugates with NK cells and stimulate NKCf release. Furthermore, the observation that U937 are resistant to LDCC suggests that NKCF or target cell binding sites for these factors may also be involved in the LDCC lytic mechanism. These results support a role for NKCF as the NK lytic mediators and suggest that TNF is not involved or else requires the presence of additional factors found in NKCf to mediate NK CMC.

G136 THE TOXIC EFFECT OF LYSOSOMOTROPIC AGENT, L-LEUCINE METHYL ESTER, AND ITS CONDENSATION PRODUCT, L-LEUCYL-L-LEUCINE METHYL ESTER, ON NK, LAK AND CTL.

Masato Yagita,* William Louden,** and Elizabeth A. Grimm,* *SNB, NINCDS, NIH, Bethesda, MD 20892 and **Dept. of M & I of the Univ. of Oklahoma, Oklahoma City, OK 73190

It has been reported that lysosomotropic agent, L-Leucine methyl ester (LeuOMe) displays a unique and selective toxicity for monocytes and NK cells. In this report the effect of LeuOMe on effector function of natural killer (NK), lymphokine activated killer (LAK), and conventional cytotoxic T lymphocytes (CTL) was examined. The treatment of peripheral blood mononuclear (PBM) cells with 5 to 40mM of LeuOMe at 37°C for 30 min caused the complete elimination of NK activity. In contrast, LAK and CTL effectors showed relative resistance to LeuOMe, with 160mM of LeuOMe needed to completely inhibit those functions, as measured in 4 hr ^{51}Cr-release assays. However, the supernatant of plastic adherent monocytes (MO) treated with 5mM of LeuOMe contained the same inhibitory activity to NK in addition to inhibiting LAK and CTL killing. These results confirmed previously reported MO-mediated-conversion of LeuOMe to the more toxic product, L-Leucyl-L-Leucine methyl ester (Leu-LeuOMe). Moreover, NK and LAK killing activity were inhibited by 100 uM of Leu-LeuOMe. Plausible explanations for the relative resistance to LeuOMe of LAK and CTL effectors in comparison with NK effectors include differential membrane diffusion and different composition of lysosome enzymes. The mechanism of inhibition of NK, LAK and CTL by Leu-LeuOMe should be further studied for the better understanding of killing mechanism of cytotoxic effectors.

G137 MORPHOLOGICAL ANALYSIS OF CYTOTOXIC LYMPHOCYTE-TARGET CELL INTERACTIONS, John R. Yannelli Susan G. Tewksbury,Gary B.Thurman,and Robert K.Oldham,Biotherapeutics,Inc.,Franklin,TN 37064,Victor H. Engelhard,James A.Sullivan,and Gerald L.Mandell,University of Virginia Medical School, Charlottesville, VA 22908.

The interaction of murine cytotoxic T lymphocyte [CTL] clones with human lymphoblastoid target cells was studied in thin preparations using high resolution cinemicrography.CTL not bound to target cells were morphologically polar,possessing a broad leading edge containing the nucleus, and a tapered tail containing a heterogeneous population of granules.The CTLmoved by extending pseudopods from the leading edge from which initial contact with a target cell was made.If the human target cell expressed the appropriate HLA antigen,the CTL rounded up and the nucleus moved away from the zone of contact to be replaced by the cytoplasmic granules. Redistribution of the granules was complete as early as 10 minutes after initial contact.These morphological changes did not occur when the CTL made contact with target cells bearing inappropriate HLA antigens.Using Nomarski optics,an apparent fusion of 2 CTL cytoplasmic granules in the vicinity of the contact area was observed 4 minutes after binding.These data provide direct evidence for the occurrence of a reorientation of the CTL cytoplasmic contents during the administration of the lethal hit and suggest exocytosis as a mechanism of granule transfer.Current studies involve the generation and morphological characterization of human "Lymphokine Activated Killer" [LAK] cells to determine the nature of the interaction between LAK cells and tumor targets.Similarities and differences with the CTL system are being analyzed and implications of these findings to the relationship between CTL and LAK cells will be discussed.

G138 EVIDENCE FOR CHOLINE RECOGNITION BY GRANULE CYTOLYSIN OF CYTOTOXIC LYMPHOCYTES: CROSS-REACTION WITH ANTI-TEPC-15 ANTI-IDIOTYPIC ANTIBODIES Cheung C. Yue, James J. Kenny*, Jan Cerney†, and Pierre A. Henkart, National Cancer Institute, Bethesda, MD 20892, *USUHS, Bethesda, MD 20814, and †University of Texas Medical Branch, Galveston, TX 77550.

We and others have reported the isolation of a potent lytic activity, termed cytolysin, from cytoplasmic granules of cytotoxic lymphocytes. As expected if the soluble protein cytolysin inserts into a target membrane to form pores, lipids are potent inhibitors of cytolysin activity with 50% inhibition at ~1 μM. In testing soluble lipid headgroup analogues, phosphocholine (PC) gave 50% inhibition at 1.4 mM, while the closely related phosphoethanolamine was a hundred fold less inhibitory. Since some non-antibody PC-binding proteins have been shown to express epitopes which cross-react with idiotopes on the PC-binding myeloma TEPC-15 (T15), LGL tumor granule cytolysin was tested for similar cross-reactive epitope(s). IgG of rabbit anti-T15 anti-idiotypic antibody (anti-Id) specifically inhibited the hemolytic activity of cytolysin, and reacted with partially purified cytolysin by ELISA. Two out of 6 murine anti-Id monoclonals inhibited cytolysin activity and reacted with partially purified cytolysin by ELISA. Using the rabbit anti-Id, cytolysin was immunoprecipitated from whole cell lysates of ^{35}S-methionine labelled LGL tumor cells and cloned CTL. Finally, rabbit antibodies raised against purified cytoplasmic granules from LGL tumors specifically reacted with a T15-idiotype bearing myeloma by ELISA. We propose that a component of the interaction between cytolysin and a cell membrane is the recognition of the choline headgroups of membrane lipids by a binding site related to the T15 idiotype.

Immunotoxins; Transmembrane Signalling; Mechanism of Cytotoxicity by Effector Cells and Products

G139 RING FORMATION DETECTED ON TARGET CELL MEMBRANES LYSED BY ANTIBODY AND COMPLEMENT BUT NOT BY CYTOLYTIC T LYMPHOCYTES. Gideon Berke and Dalia Rosen, Department of Cell Biology, The Weizmann Institute of Science, PO Box 26, Rehovot 76100, Israel.
The mechanism whereby specifically sensitized cytolytic T lymphocytes (CTL) or natural killer (NK) cells lyse target cells (TC) has been equated with that of antibody + complement (Ab+C) That is, the perforation of the TC membrane by ring-shaped structures (I.D. 10-15 nm) of effector cell origin, structurally and functionally analogous to those produced by the membrane attack complex of the C system. (Recently, however, the precise role of these structures even in the C system has been questioned since proteolysis of C_9 allows lysis, although impairing poly(C_9) and circular lesion formation). We have confirmed the presence of typical, ring-shaped structures on 118/343 membrane fragments derived from nucleated TC following Ab+C attack, using negative staining EM. Examining NK activity of unfractionated mouse spleen cells or human peripheral blood lymphocytes, revealed similar ring structures in 10/280 and 2/130 membrane fragments examined, respectively; however, a correlation between rings and lysis has not been shown and involvement of C complnents has not been excluded. In contrast, we have been unable to observe similar rings on over 2000 membrane fragments, examined individually, following attack by *in vivo* primed, specifically sensitized, alloreactive CTL or by polyclonally activated CTL in lectin-(Con A)-dependent cytotoxicity. These results do not support recent claims of a common lytic mechanism employed by specifically sensitized CTL and by Ab+C and suggest instead, a distinct pathway for CTL not involving membrane perforation by C-like 'ring structures. Our results do not preclude involvement of cytolytic granules nor secreted material thereof in the lytic process.

G140 REGULATION OF QUIESCENT B LYMPHOCYTE ACTIVATION VIA MULTIPLE TRANSMEMBRANE SIGNALING MECHANISMS. John C. Cambier, Zheng Z. Chen, John T. Ransom, Michael G. Klemsz, Leslie K. Harris, Victoria M. Sandoval and M. Karen Newell. Dept. of Medicine, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO. 80206

Small B lymphocytes respond in qualitatively disparate ways to anti-immunoglobulin/antigen, B cell stimulatory factor 1, Interferon γ, and anti-MHC class II antigen antibodies. Here we report comparative analysis of transmembrane signaling mechanisms utilized by membrane immunoglobulin; I-A/E and BSF1 receptors. Results indicate that signal transluction by these receptors occurs by quite distinct mechanisms. Once bound by ligand, membrane IgM and IgD activate a $PtdInsP_2$ hydrolysis cascade. BSF1 receptors do not utilize this mechanism, although surprisingly they activate I-A gene expression more efficiently than mIg binding ligands. Finally, when bound by soluble ligands, I-A and I-E transduce negative signals which lead to suppression of anti-Ig responses. Transduction of this signal appears not to involve PtdIns metabolism but has a pronounced effect on PKC compartmentalization, leading to PKC translocation to the nucleus. These findings illustrate that multiple molecular "second messengers" regulate B cell activation and that the ultimate biologic response of cells may in many instances be a function of interactive aspects of the signaling machinery.

G141 PURIFICATION OF RICIN A AND B CHAINS AND CHARACTERIZATION OF THEIR TOXICITY,' R.J. Fulton, D.C. Blakey, P.P. Knowles, J.W. Uhr, P.E. Thorpe and E.S. Vitetta, University of Texas Health Science Center at Dallas, Dallas, Texas 75235 and the Imperial Cancer Research Fund Laboratories, London, England

Methods used for the purification of ricin A chain, to be used in the preparation of immunotoxins, vary among laboratories. In many cases, a single affinity or ion exchange procedure has been used to separate the B chain from the A chain after reduction of the interchain disulfide bond. In this report, we describe a more extensive purification scheme employing a series of alternating affinity and ion exchange procedures which yields fully active A and B chains that are minimally cross-contaminated. These materials have been tested for nonspecific toxicity in mice and on a variety of cell types *in vitro*. The results demonstrate that meticulous removal of cross-contaminating chains from both the A chain and B chain preparations reduces the *in vitro* and *in vivo* toxicity of both chains. In addition, the results demonstrate that ammonium chloride markedly enhances the toxicity of ricin B chain and A chain preparations that are contaminated with B chain, but does not enhance the cytotoxicity of purified ricin A chain or an immunotoxin prepared with the A chain.

G142 PERIPHERAL BENZODIAZEPINES, CELL GROWTH AND PROTO-ONCOGENE EXPRESSION, James Morgan and Sydney Spector, Department of Physiological Chemistry and Pharmacology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, New Jersey 07110.

Peripheral type benzodiazepines (P-BZDs) interact with nanomolar affinities with a binding site present in most non-neuronal cells. This P-BZD site is pharmacologically distinct from the diazepam receptor of brain and is particularly abundant in tumor cells where it exhibits properties characteristic of classical receptors. P-BZDs that bind avidly to the P-BZD site are potent antimitotic agents (1) and are cytotoxic for rapidly proliferating cells. Besides their antimitotic potential P-BZDs can induce differentiation in several tumor cell types (2,3). This activity appears to be mediated by a site quite distinct from the P-BZD binding site described above. In addition this second locus of action can be shown to be linked to the expression of the c-fos proto-oncogene in one tumor system (4). Thus we have in hand a class of compounds that act as probes for the transduction mechanisms that control cell growth and differentiation. Clearly an elucidation of the function of the P-BZD binding site will greatly aid our understanding of such controlling mechanisms. Presently we are engaged in defining the signals that couple BZDs to genomic events and are cloning the gene for the P-BZD binding site which may represent a substrate for the future investigation of cytotoxins

(1)P.N.A.S. (1984) 81;753. (2) P.N.A.S. (1984) 81;3770. (3) P.N.A.S. (1985) 82;5223. (4)Science (1985) 229;1265.

G143 CHARACTERIZATION OF A PROTEIN 4.1 ANALOG FROM MURINE B LYMPHOCYTES, Gary R. Pasternack and Asoka I. Katumuluwa, Dept. of Pathology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Numerous studies have established that B lymphocyte surface immunoglobulin exists in the membrane complexed with components of the underlying membrane skeleton, and that cross-linking of surface receptors results in concomitant redistribution of membrane skeletal components. The focus of our laboratory has been 1) to isolate and characterize sIg-membrane skeletal complexes from activatable murine B cell lines, and 2) to purify and characterize individual membrane skeletal components from these cells. We report the isolation of a novel 40 kDa phosphoprotein expressed by four murine B cell lines which is related to erythrocyte protein 4.1. The 40 kDa analog, initially identified by immunoblotting with affinity-purified antibody to human red cell protein 4.1, was purified by coprecipitation from detergent lysates of B cell lines using stably cross-linked myosin filaments, followed by elution in 1 M KCl and DEAE cellulose chromatography. This phosphoprotein occurs in multiple isoforms with pI's ranging between 6.8 and 4.5, all possessing identical two-dimensional peptide maps, and seems to share features in common with the acidic portion of the 80 kDa erythrocyte form. These findings are intriguing, since in red cells, protein 4.1 seems to regulate the attachment of the membrane skeleton to integral membrane proteins.

Supported by grants from the Andrew W. Mellon Foundation and the Anna Fuller Fund.

G144 EFFECT OF DIFFERENT HETEROBIFUNCTIONAL CROSSLINKERS ON ACTIVITY AND PHARMACOKINETICS OF ANTIBODY CONJUGATES WITH COBRA VENOM FACTOR. E.C. Petrella, S.D. Wilkie, A.H. Grier, and C.-W. Vogel. Depts. Biochem. and Med. and Vincent T. Lombardi Cancer Center, Georgetown University, Washington, DC 20007.

Seven heterobifunctional crosslinking reagents with different sulfhydryl-reactive groups (succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), -N-maleimidobutyryl-N-hydroxysuccinimide ester (GMBS), m-maleimidobutyryl-hydroxysuccinimide ester (MBS), succinimidyl-4-(p-maleimidophenyl)-butyrate (SMPB), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), N-succinimidyl(4-iodoacetyl)-aminobenzoate (SIAB), iodoacetyl-N-hydroxysuccinimide ester (IAHS)) were used for the synthesis of antibody conjugates with the complement-activating cobra venom factor (CVF). Modification of CVF with SPDP did not affect its hemolytic activity while the other six reagents caused 50 $\geq$ 95% inhibition. Inhibition of CVF correlated with intramolecular crosslinks due to the lack of specificity of the sulfhydryl-reactive groups of these six reagents. Pharmacokinetic studies with derivatized CVF, derivatized IgG, and antibody-CVF conjugates were performed in BALB/c mice. The plasma disappearance of the proteins followed first order kinetics. Major differences were observed for proteins derivatized or conjugated with different reagents. We found that crosslinkers a) with a halide group cause covalent binding to plasma proteins leading to increased plasma half-times (IAHS, SIAB), b) with an aromatic ring system result in rapid removal from the circulation (MBS, SMPB), and c) with a disulfide bond are subject to moderate cleavage (SPDP). These studies demonstrate that heterobifunctional crosslinking reagents have major impact on the activity and pharmacokinetics of antibody conjugates. (Supported by NIH grants CA 35525 and CA 01039).

G145 INDUCTION OF THE C-MYC PROTOONCOGENE FOLLOWING ANTIGEN BINDING TO HAPTEN-SPECIFIC B CELLS. E.C. Snow, J. Fetherston, & S. Zimmer. Univ. of Ky.,Lexington,KY 40536. Considerable controversy has centered on the role that the surface immunoglobulin (sIg) receptor for antigen plays during the induction of B cell activation. Stimulation by anti-Ig reagents has been shown to activate G_0 B cells to enter the cell cycle. The binding of thymus-dependent antigens to hapten-specific B cell populations apparently does not result in the movement of the antigen-binding cells (ABC) into the G_1 state of the cell cycle. However, we have recently demonstrated that antigen binding to such hapten-specific B cells does result in the initiation of the membrane phosphatidylinositol cycle. In the present experiments, hapten-specific B cells (80-90% ABC, 99% in G_0) were incubated with either the correct hapten-carrier conjugate, with the carrier protein, or only media for 2 hours at 37°C. At that time, total cellular RNA was isolated and subsequently analyzed by either dot blots or Northern gel techniques. The blots were probed with a [^{32}P]-c-myc SstI-XhoI fragment. The results indicate that hapten carrier stimulation of the hapten-specific B cells induces enhanced transcription of the c-myc gene. These observations lend further support to the premise that antigen binding to the sI g receptor results in the transduction to the cell of important signals and implicates the active participation of sIg during the process of antigen-mediated B cell activation.

G146 LYSIS OF FRESH HUMAN TUMOR CELLS BY AUTOLOGOUS LARGE GRANULAR LYMPHOCYTES AND MACROPHAGES: INVOLVEMENT OF CYTOTOXIC FACTORS. Atsushi Uchida and Eva Klein, Karolinska Institutet, S-104 01 Stockholm, Sweden.

When blood and tumor-associated large granular lymphocytes (LGL) and macrophages from cancer patients were cocultured with autologous freshly isolated tumor cells, they released soluble factors, natural killer cytotoxic factors (NKCF) and monocyte cytotoxic factors (MCF), respectively. that killed autologous and allogeneic fresh tumor cells as well as K562 cells. Treatment of fresh tumor cells with actinomycin D or interferon (IFN) resulted in a reduction of their capacity to stimulate the release of cytotoxic factors from autologous LGL, but not from autologous macrophages, and of their susceptibility to lysis by autologous LGL. Actinomycin D-treated target cells, however, increased the sensitivity to the cytotoxic factors. The presence of IFN in the cytotoxicity assay also enhanced the lytic activity of both cytotoxic factors. Treatment of fresh tumor cells with the streptococcal preparation OK432 induced them to become more susceptible both to direct cell-mediated and cytotoxic factor-dependent lysis. When LGL and macrophages were pretreated with OK432, they showed an increase in their ability to kill directly autologous tumor cells and to release the cytotoxic factors in response to autologous tumor cells. Stimulation with OK432 alone of monocytes and macrophages was effective in inducing cytotoxic factors, while IFN had no such effects. These data may indicate the involvement of soluble cytotoxic factors in the autotumor killing by LGL and macrophages and suggest that their activity is regulated in different ways by each biological response modifire.