SUPPRESSION OF AMPHOTERICIN B METHYL ESTER INDUCED GROWTH STIMULATION IN INTRASPECIFIC MOUSE SOMATIC CELL HYBRIDS

PAUL B. FISHER*, VERNON BRYSON and CARL P. SCHAFFNER

Waksman Institute of Microbiology, Rutgers University
New Brunswick, N. J. 08903, U.S.A.

(Received for publication November 8, 1976)

Amphotericin B methyl ester (AME), the chemically modified derivative of amphotericin B, induced a concentration-dependent growth stimulatory effect on B82 mouse cells as indicated by increased 24- and 72-hour viable cell number, growth rate and DNA and RNA synthesis. In contrast, AME was not growth promoting toward RAG mouse cells or B82-RAG somatic cell hybrids, while hybrid cells exhibited the increased AME resistance pattern of B82 parental cells. A dissociation between the phenotypic expression of growth stimulation and polyene sensitivity was demonstrated in intraspecific mouse hybrids.

Previous studies have indicated that the structurally modified polyene macrolide antibiotic, amphotericin B methyl ester (AME)[1,2] is: (a) selectively toxic toward specific human and mouse tumor cells in comparison with cells derived from normal tissue[3]; and (b) growth stimulating toward L-M (mouse) and Vero (monkey) cells.[4] In the present study we investigated the effect of genome interaction on: (a) the expression of polyene macrolide antibiotic sensitivity in intraspecific mouse hybrids formed between more resistant B82 cells of normal origin[5] and sensitive tumor derived RAG cells[6]; and (b) the phenotypic expression of AME induced growth stimulation in hybrids formed between AME responsive B82 and non-responsive RAG parental cells.

Materials and Methods

Polyene Macrolide Antibiotic:

Amphotericin B methyl ester[1,2] was synthesized and supplied by Dr. WITOLD MECHLINSKI, Waksman Institute of Microbiology, Rutgers University, New Brunswick, N. J. Desired concentrations of AME were prepared in dimethyl sulfoxide (DMSO) immediately prior to use.

Cell Culture Systems:

The B82 and RAG parental cell lines utilized in this study were of mouse origin. The B82 cell line is a subline of mouse L fibroblasts which lacks the enzyme thymidine kinase (TK^-) and is resistant to 30~300 μg/ml of 5-bromodeoxyuridine[5]. This cell type was kindly supplied by Dr. RICHARD W. ERBE, Massachusetts General Hospital, Boston, Mass. The RAG cell line was originally derived from a cloned population of renal adenocarcinoma cells, lacks the enzyme hypoxanthine-guanine phosphoribosyltransferase ($HGPRT^-$) and is resistant to 8-azaguanine[6]. This cell line was a gift from Dr. FRANK H. RUDDLE, Yale University, New Haven, Conn. B82 and RAG cells were grown at 37°C in DULBECCO's modified EAGLE's minimum essential medium (DMEM) supplemented with 15% fetal calf serum (FCS), non-essential amino acids (NEAA), L-glutamine and 50 μg/ml of gentamicin (Microbiological Associates)—referred to as complete growth medium. B82-RAG somatic cell hybrids were formed by lysolecithin induced fusion[7] and selected in complete growth medium supplemented with 1×10^{-4}M hypoxanthine, 4×10^{-7}M aminopterin and 1.6×10^{-5}M thymidine (DMEM-HAT)[8] as previously described[9,10]. Clones of hybrid cells selected three weeks after fusion and selection in DMEM-HAT were

* Present address: Institute of Cancer Research, Columbia University College of Physicians and Surgeons, New York, New York 10032, U.S.A.

confirmed as hybrids by chromosomal analysis and polyacrylamide gel electrophoretic analysis of lactate dehydrogenase (LDH)[9,10]. Both parental (B82 and RAG) and hybrid (B82-RAG) cells were routinely screened for possible mycoplasmal contamination[4].

Cell Viability and Growth Rate Analysis:

Cell survival studies on B82, RAG and B82-RAG (passage 4~12) cells in either the absence or presence of various concentrations of AME were performed over a 24-hour test period[3,4,9~12]. Growth rates and population-doubling times of control and treated cultures were determined between 24 and 72 hours[3,9~12]. The TCD_{50} level, the concentration of AME resulting in approximately a 50% reduction in viable cell number, as opposed to final control value, was determined after a 72-hour test period for each cell type[3,9~12]. Cell survival, growth rate and TCD_{50} studies were performed using a minimum of 3 replicate experiments. Equal numbers of cells ($2\sim4\times10^5$) in 1 ml of complete growth medium (containing 10% as opposed to 15% FCS) were inoculated into replicate 30×15 mm tissue culture plates, followed by a single administration of a 10 μl volume of AME to yield: 1~400 μg of AME per ml. AME was dissolved in DMSO. Ten counts of viable cells were performed on day 0 and 20 (10 per replicate sample) counts were performed on day 1, 2 and 3 with a haemocytometer and the trypan blue dye exclusion technique[3,9~12].

Monitoring Membrane Damage by [^{51}Cr] Release:

Immediate (15~60 minutes) and time course (2~24 hours) membrane permeability changes induced by AME in parental and 4th~12th passage hybrid cells were monitored by a modified [^{51}Cr] release assay as previously described[11,13]. Studies were performed a minimum of three times using triplicate samples. Percentage [^{51}Cr] release was expressed in relation to freeze-thawed control cultures.

Determination of DNA and RNA Synthesis in the Presence of AME:

Macromolecular synthesis of DNA and RNA in the presence of AME, 1~400 μg/ml was determined by the amount of labeled precursors, [^{3}H] thymidine or [^{3}H] uridine, incorporated into acid-insoluble material over a 24-hour (DNA) or 3-hour (RNA) test period as previously described[4]. The amount of label incorporated was expressed in relation to DMSO or untreated control cultures. Triplicate samples for each AME concentration were utilized and experiments were performed a minimum of three times.

Results

RAG cells were more sensitive than B82 or B82-RAG hybrid cells to immediate (15~60 minutes) and long range (2~24 hours) membraned amage as monitored by [^{51}Cr] release. Approximately a 50% release of [^{51}Cr] resulted after exposure of RAG cells to 150 μg/ml of AME for 15 minutes, whereas no significant (<8%) release of [^{51}Cr] resulted when B82 or B82-RAG hybrid cells were treated with 1~400 μg/ml of AME for 60 minutes. In contrast, 40~400 μg/ml of AME induced a concentration-dependent release of [^{51}Cr] from parental and hybrid cells after exposure for 12~24 hours (monitored at 2-hour intervals), whereas a similar pattern of release as found after 15~60 minutes was evident in all three cell types treated with 40~400 μg/ml of AME for periods <12 hours.

AME was selectively toxic toward RAG cells and was found to be 50 times more toxic toward this cell type in comparison with B82 or B82-RAG (passage 4~10) cells as indicated by 72-hour viability (TCD_{50}). Approximately a 50% reduction in 72-hour viable cell number resulted when B82 or B82-RAG cells were exposed to 250 μg/ml of AME, whereas a similar reduction in viable cell number resulted after 72-hour exposure of RAG cells to 5 μg/ml of AME.

The increased sensitivity of RAG cells to AME was clearly evident after both 24 (Fig. 1) and 72 (Fig. 2) hours. A comparison of the effect of different concentrations of AME on the growth rate (population doubling time) of parental and hybrid cells again indicated the increased sensitivity of RAG cells to this polyene antibiotic. AME levels of 5~10 μg/ml resulted in a reduced growth rate of RAG

Fig. 1. Effect of amphotericin B methyl ester (AME) on 24-hour survival of B82, RAG and B82-RAG cells.

Equal number of B82, RAG or B82-RAG cells, $2\sim4\times10^5$, in replicate 30×15 mm tissue culture plates were either untreated or treated with $1\sim400$ μg/ml of AME added at the initial time of cell plating (Time 0). Survival is expressed in relation to untreated or DMSO treated controls (100%).

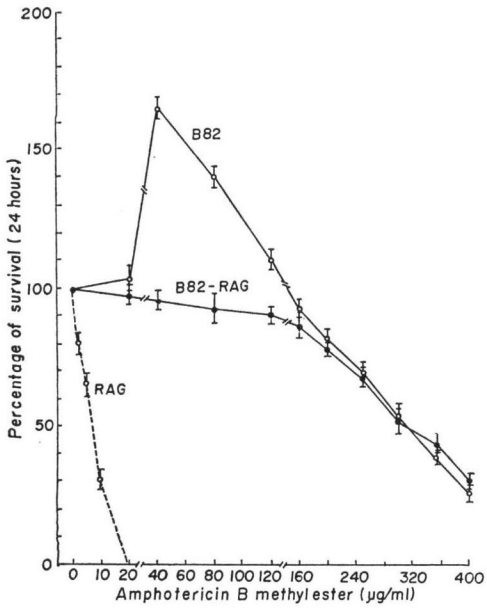
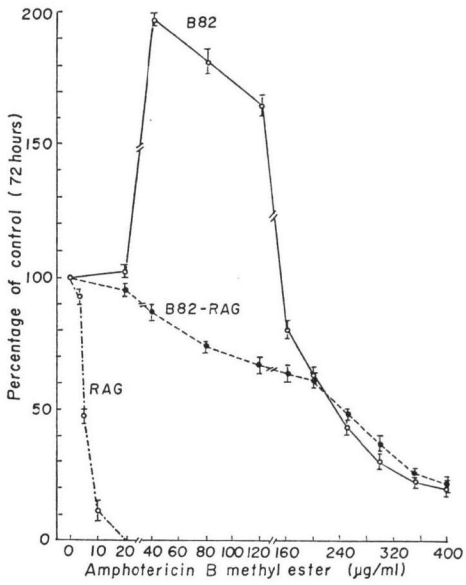


Fig. 2. Effect of amphotericin B methyl ester (AME) on 72-hour growth of B82, RAG and B82-RAG cells.

Equal number of B82, RAG or B82-RAG cells, $2\sim4\times10^5$, in replicate 30×15 mm tissue culture plates were either untreated or treated with $1\sim400$ μg/ml of AME (added at the initial time of cell plating) for 72 hours. Results are expressed as percentage of controls (100%).



cells, whereas >200 μg/ml was required to elicit a similar response in B82 or B82-RAG cells. RAG cells also differed from B82 and B82-RAG cells in their ability to survive for 24~72 hours after a single exposure to higher concentrations of AME. Total cell death (<1% survival) resulted in 24~48 hours after treatment of RAG cells with $\geq$20 μg/ml of AME, whereas B82 and B82-RAG cells survived after 72-hour exposure to 400 μg/ml of AME.

AME induced a pronounced stimulatory effect on B82 cell growth as evidenced by increased 24- and 72-hour viable cell number, growth rate and incorporation of [^{3}H] thymidine and [^{3}H] uridine into DNA and RNA respectively. AME concentrations of 40~120 μg/ml resulted in a 1.6~2.2-fold increase in 72-hour viable cells in comparison with control cultures. The average population doubling time of B82 cells was reduced from 28.6 ± 2.1 hours (control) to a low of 21.3 ± 0.9 hours after treatment with 40~120 μg/ml of AME. A concentration-dependent increase in both DNA (Fig. 3) and RNA (Fig. 4) synthesis was also evident after treatment of B82 cells with 40~120 μg/ml of AME. In contrast, no stimulatory effect was evident in RAG or hybrid cells treated with 1~400 μg/ml of AME for 24 or 72 hours.

Both parental and hybrid cells were found to be free of mycoplasmal contamination as determined by tube cultures (1~3 weeks) and agar plates (1~3 weeks)[4].

Fig. 3. DNA synthesis in B82, RAG and B82-RAG cells in the presence of AME.

[^{3}H] Thymidine was added to B82, RAG and B82-RAG cell cultures in the presence of 1~400 μg/ml of AME. Incorporation of the radioactivity into TCA precipitable DNA after 24 hours was determined.

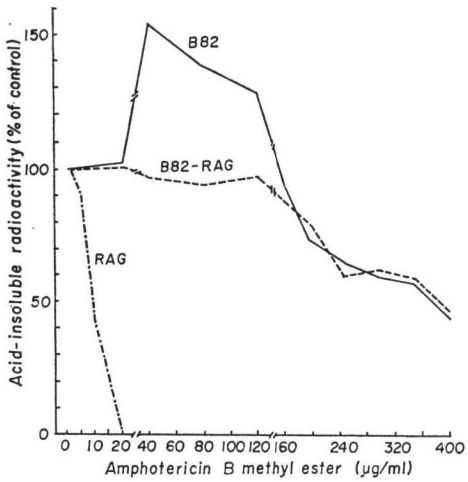
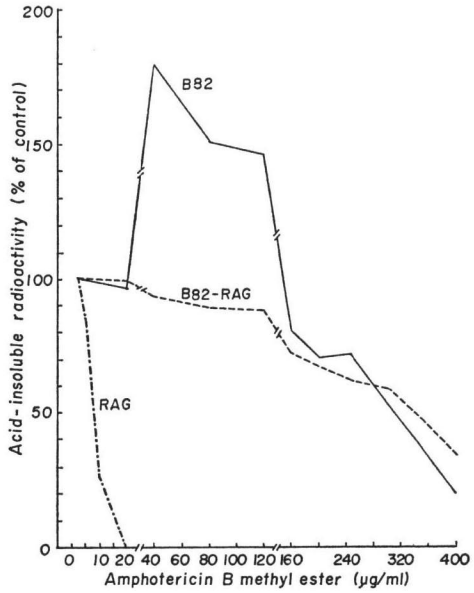


Fig. 4. RNA synthesis in B82, RAG and B82-RAG cells in the presence of AME.

[^{3}H] Uridine was added to B82, RAG and B82-RAG cell cultures in the presence of 1~400 μg/ml of AME. Incorporation of the radioactivity into TCA precipitable RNA after 3 hours was determined.



Discussion

Somatic cell hybridization has proven to be an extremely useful procedure for investigating the effect of genome interaction on the expression of diverse properties in eukaryotic cells[14~16]. However, few studies have been aimed at elucidating the relationship between antibiotic sensitivities in hybrid cells formed between parental cells exhibiting differential responses to these agents[9,10,17,18]. Studies in our laboratory[3,4,9~11,17] have indicated major differences in the innate sensitivity of eukaryotic cells to various polyene macrolide antibiotics, including AME. In the present study neither AME sensitivity nor growth stimulation were expressed in early passage B82-RAG intraspecific mouse somatic cell hybrids. Hybrid cells reflected the increased AME resistance of B82 parental cells, but did not exhibit growth stimulation as found with B82 parental cells.

Polyene macrolide antibiotic toxicity has most often been equated with membrane permeability alterations which result from polyene macrolide-cholesterol interactions[19~22]. In the present study, AME did not induce significant levels of [^{51}Cr] release ($<8\%$) after treatment of B82, RAG or B82-RAG cells with 1~120 μg/ml for time periods <12 hours, whereas a concentration-dependent release in [^{51}Cr] was evident in parental and hybrid cells treated with AME for periods exceeding 12 hours. In contrast, studies using the parent antibiotic amphotericin B (AB) have demonstrated marked changes in [^{51}Cr] permeability after only 15 minutes exposure to 1~3 μg/ml[11]. These findings suggest that AME induced changes in [^{51}Cr] membrane permeability, unlike those induced by AB, are a consequence of membrane modifications resulting from extended exposure (≥ 12 hours) of cells to this agent. It is therefore feasible that both AME induced growth stimulation and toxicity may be consequences of the same phenomenon—polyene macrolide induced changes in membrane permeability.

The ability of diverse agents, including serum[23], transforming viruses[24,25] and other mitogenic substances[26], to stimulate the growth of eukaryotic cells has been found to correlate with an increase in the transport of low molecular weight nutrients into cells. Growth stimulation does not appear to

be a random process characteristic of all cells, but a selective process influencing only specific types and strains of cells[27]. AME appears to be capable of selective action[4]. It stimulates L-M and Vero cell growth[4], but does not stimulate the growth of other cell types[3,4,9~11]. AME (40~120 μg/ml) modulated stimulation of B82 cell growth may therefore occur because B82 cells are transport deficient and AME mediated permeability alterations facilitate the uptake of small molecular weight nutrients from the culture medium. The possibility that AME induced growth stimulation results by altering physiological functions of B82 cells, *i.e.* metabolic stimulation, seems unlikely because: (1) AME is not universally growth stimulating toward all cell types[3,4,9~11]; and (2) the major site of polyene macrolide interaction appears to be the cell membrane specifically sterol(s) (cholesterol) components[19~22]. Stimulation of cell growth as a result of elimination of fungal or mycoplasmal contamination also seems unlikely since B82 cells (as well as RAG and B82-RAG) were free of contamination.

The suppression of growth stimulation in B82-RAG hybrids does not appear to correlate with the loss of specific B82 chromosomes, since chromosome analysis of early passage hybrid cells has demonstrated the presence of a complete genome equivalent from B82 and RAG cells[10]. Total suppression of the RAG or B82 genotype in hybrid cells can be ruled out for the following reasons: (a) the growth of hybrid cells in HAT medium, which requires the production of both hypoxanthine-guanine phosphoribosyl transferase (B82) and thymidine kinase (RAG); (b) the production of hybrid lactate dehydrogenase isoenzymes, most likely resulting from B82 and RAG subunit rearrangement in the hybrids[9,10]; and (c) the production of cell surface glycoprotein species of B82 and RAG origin in hybrids[28]. The mechanism involved in the suppression of AME induced growth stimulation in B82-RAG hybrids may be a consequence of differences in the surface composition of these cells, specifically the amount, type or molecular orientation of sterol(s) in the membranes of hybrid cells. Support for this hypothesis comes from studies by FISHER *et al.*[28] of cell surface glycoproteins from B82-RAG hybrid cells. Electrophoretic analysis of [^{3}H]-glucosamine labeled surface glycoproteins from B82, RAG and B82-RAG hybrid cells has indicated alterations in the composition of these membrane components in hybrids.

Cell types of diverse origin have been found to exhibit major differences in sensitivity to AME[3,4,9,11]. In the present study, RAG cells were 50 fold more sensitive to the cytotoxic effect of AME than B82 or B82-RAG cells (Figs. 1 and 2). The reason for the innate differential toxicity of AME toward RAG and B82 cells may be variations in the membrane composition of parental cells or the ability of cells to repair AME induced membrane modifications. The B82 level of resistance in B82-RAG cells may indicate that the transport defect of B82 cells, which may be related to membrane structure, has been corrected as a consequence of B82 and RAG genome interactions. The dissociation of polyene macrolide antibiotic toxicity and growth stimulation is suggestive of differences in the genetic regulation of these processes. By using hybrid systems which selectively lose specific chromosomes of one of the parental cells[14~16] it may ultimately be possible to determine which chromosome(s) are involved in determining the phenotypic expression of polyene macrolide sensitivity and growth stimulation in eukaryotic cells.

Acknowledgements

This investigation was supported in part by NIH Grant No. AI-2095, NIH Grant No. AI-12602, and NIH Training Grant No. GM507 from the National Institutes of Health.

The authors express their appreciation to Dr. WITOLD MECHLINSKI for the availability of amphotericin B methyl ester (AME). The authors also acknowledge Drs. RICHARD W. ERBE and FRANK H. RUDDLE for gifts of cell lines employed in this study.

References

1) MECHLINSKI, W. & C. P. SCHAFFNER: Polyene macrolide derivatives. I. N-Acylation and esterification reactions with amphotericin B. J. Antibiotics 25: 256~258, 1972
2) SCHAFFNER, C. P. & W. MECHLINSKI: Polyene macrolide derivatives. II. Physical-chemical properties of polyene macrolide esters and their water soluble salts. J. Antibiotics 25: 259~260, 1972
3) FISHER, P. B.; N. I. GOLDSTEIN, D. P. BONNER, W. MECHLINSKI, V. BRYSON & C. P. SCHAFFNER: Toxicity

of amphotericin B and its methyl ester toward normal and tumor cell lines. Cancer Res. 35: 1996~1999, 1975

4) FISHER, P. B.; N. I. GOLDSTEIN, D. P. BONNER, W. MECHLINSKI, V. BRYSON & C. P. SCHAFFNER: Stimulatory effect of amphotericin B methyl ester on the growth of L-M and Vero cells. J. Antibiotics 28: 896~902, 1975
5) LITTLEFIELD, J. W.: Studies on thymidine kinase in cultured mouse fibroblasts. Biochim. Biophys. Acta 95: 14~22, 1965
6) KLEBE, R. J.; T. CHEN & F. H. RUDDLE: Controlled production of proliferating somatic cell hybrids. J. Cell Biol. 45: 74~82, 1970
7) KEAY, L.; S. A. WEISS, N. CIRULIS & B. W. WILDI: Lysolecithin-induced fusion of fibroblasts. In Vitro 8: 19~25, 1972
8) LITTLEFIELD, J. W.: Selection of hybrids from mating of fibroblasts *in vitro* and their presumed recombinants. Science 145: 709~710, 1964
9) FISHER, P. B.: Polyene sensitivity of intraspecific and interspecific somatic cell hybrids. Ph. D. Thesis, Rutgers University, pp. 1~180, 1974
10) FISHER, P. B. & V. BRYSON: Toxicity of nystatin and its methyl ester toward parental and hybrid mammalian cells. In Vitro, In press
11) FISHER, P. B.; N. I. GOLDSTEIN, V. BRYSON & C. P. SCHAFFNER. Reduced toxicity of amphotericin B methyl ester (AME) *vs.* amphotericin B and Fungizone in tissue culture. In Vitro 12: 133~140, 1976
12) BONNER, D. P.; P. B. FISHER, N. I. GOLDSTEIN, W. MECHLINSKI, V. BRYSON & C. P. SCHAFFNER: Effect of fetal bovine serum on polyene antibiotic cytotoxicity and antifungal activity. In Vitro 12: 399~404, 1976
13) FISHER, P. B.: "Quantitation of membrane damage by (^{51}Cr) release". *in* Techniques, Methods and Procedures for Cell, Tissue and Organ Culture (*Eds.* V. J. EVANS, V. P. PERRY & M. M. VINCENT), Tissue Culture Association Manual, In press
14) HARRIS, H.: Cell Fusion. Harvard University Press, Cambridge, Massachusetts, 1970
15) EPHRUSSI, B.: Hybridization of Somatic Cells. Princeton University Press, Princeton, New Jersey, 1972
16) DAVIDSON, R. L. & F. DE LA CRUZ: Somatic Cell Hybridization. Raven Press, New York, 1974
17) FISHER, P. B.; V. BRYSON, D. P. BONNER, W.MECHLINSKI & C. P. SCHAFFNER: Growth stimulatory effect of amphotericin B methyl ester (AME) on B82 mouse cells and the absence of stimulation in B82/RAG somatic cell hybrids. Annual Meeting American Society for Microbiology, Chicago, Illinois, Abst. M34, 1974
18) AMATI, P. & C. LAGO: Sensitivity to amphotericin B of *in vitro* established cell lines. Nature 247: 466~469, 1974
19) CASS, A.; A. FINKELSTEIN & V. KRESPI: The ion permeability in thin lipid membranes by the polyene antibiotics nystatin and amphotericin B. J. Gen. Physiol. 56: 100~124, 1970
20) KINSKY, S. C.: Antibiotic interaction with model membranes. Ann. Rev. Pharmacol. 10: 119~142, 1970
21) MEDOFF, G.; C. N. KWAN, D. SCHLESSINGER & G. S. KOBAYASHI: Potentiation of rifampicin, rifampicin analogs and tetracycline against animal cells by amphotericin B and polymyxin B. Cancer Res. 33: 1146~1149, 1973
22) DEKRUIJFF, B.; W. J. GERRITSEN, A. OERLEMANS, R. A. DEMEL & L. L. M. VAN DEENEN: Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* and lecithin liposomes. I. Specificity of membrane permeability changes induced by polyene antibiotics. Biochim. Biophys. Acta 339: 30~43, 1974
23) SEFTON, B. & H. RUBIN: Stimulation of glucose transport in cultures of density-inhibited chick embryo cells. Proc. Nat. Acad. Sci. U.S.A. 68: 3154~3157, 1971
24) ISSELBACHER, K.: Increased uptake of amino acids and 2-deoxy-D-glucose by virus transformed cells in culture. Proc. Nat. Acad. Sci. U.S.A. 69: 585~589, 1972
25) HATANAKA, M. & H. HANAFUSA: Analysis of a functional change in membrane in the process of cell transformation by ROUS sarcoma virus; alteration in the characteristics of sugar transport. Virology 41: 647~652, 1970
26) PETERS, J. H. & P. HAUSEN: Effect of phytohemagglutinin on lymphocyte membrane transport. I. Stimulation of uridine uptake. Europ. J. Biochem. 19: 502~508, 1971
27) TEMIN, H. M.; R. W. PIERSON, Jr. & N. C. DULAK: *in* "Growth Nutrition and Metabolism of Cells in Culture". Vol. 1 (*Eds.* G. H. ROTHBLATT & V. J. CRISTOFALO), Academic Press, New York, 1972
28) FISHER, P. B.; N. I. GOLDSTEIN & V. BRYSON. Electrophoretic analysis of surface glycoproteins from intraspecific and interspecific somatic cell hybrids. In Vitro 10: 339, 1974