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Interleukin 2 in Cell-Mediated Immune Responses

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The lymphokine Interleukin 2 (IL2) restores T cell responses in a number of in vitro systems where immunogenicity has been compromised. UV irradiation of the stimulating allogeneic cells in a mixed leukocyte culture eliminates the production of cytotoxic T lymphocytes and greatly reduces the DNA synthesis response. IL2 restores both parameters. UV-irradiated stimulators are also unable to induce the normal production of IL2 which is observed in a mixed leukocyte culture. The cytotoxic activity of allogeneically stimulated thymocytes is almost completely lost within 24 hours after removal of IL2 at 5 days, indicating that the lymphokine is continuously required to maintain CTL. Thymocytes in 4-day cultures do not adsorb IL2 unless they are simultaneously activated with a mitogen. Finally, IL2 does not adequately restore a secondary response to the purified protein derivative of tuberculin (PPD) in adherent-cell-depleted cultures, indicating that macrophages, in addition to being required for IL2 production, have other functions. These probably include the presentation of soluble antigens to responding cells.

Key words: Interleukin 2, cytotoxic lymphocytes, UV irradiation

The generation of specific cellular immunity involves a number of cellular components. Although direct cell-cell interactions are important, some interactions can apparently be replaced with soluble mediators. In this paper evidence is presented that the mediator of immune functions, Interleukin 2 (IL2) is involved in the generation of specific cytotoxic T lymphocytes (CTL). Manipulations which abrogate the production of IL2 compromise this response, and addition of the factor restores it.

Abbreviations: A cell, cells adherent to nylon wool; Con A, concanavaline A; CMI, cell-mediated immunity; CTL, cytotoxic T lymphocyte; I11 and IL2, Interleukins 1 and 2; LAF, lymphocyte activating factor, same as IL1; 2-Me, 2 mercaptoethanol; MLC(R), mixed leukocyte culture (reaction); PPD, purified protein derivative of tuberculin; TCGF, T cell growth factor, same as IL2.

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A number of lymphokines have been compared and found to be biochemically and biologically indistinguishable. One such entity, variously called costimulator [1], TCGF [2], killer cell helper factor [3], thymocyte stimulating fact [4], or thymocyte mitogenic factor [3], and exhibiting activity described as T cell replacing factor for AFC responses [5], has now been designated Interleukin 2 (IL2) [6]. This designation reflects the fact that these various biological activities appear to reside in the same molecule(s). In mice, IL2 is a protein or glycoprotein of MW about 30,000, Ia^-, and nonspecific for H-2 of the responding cell or for the antigen [7, 8]. It is produced by T cells which are helpers for CTL generation [8, 9] in response to mitogenic or strong antigenic stimulus plus the factor IL1. IL1, in turn, is generated from suitably activated macrophages [8]. Thus, IL2 appears to fit the requirements for a T helper cell product involved in regulation of CTL responses.

CTL responses in vitro can be compromised in a number of ways. Treatment of stimulating allogenetic cells with glutaraldehyde [10], UV irradiation [11, 12], or metabolic inhibitors [13] makes them non-immunogenic, although antigenicity can still be demonstrated. Helper factors, whose production is UV-sensitive, can overcome UV-induced deficiencies in CTL generation [14, 15]. We have previously shown that IL2 purified through column chromatography and isoelectric focusing can restore immunogenicity to glutaraldehyde-treated stimulator cells [16], a result consistent with the observations of others on UV-treated cells [11, 12]. In the present work, UV irradiation has been used to manipulate the required helper function in CTL generation. A correlation will be demonstrated between the ability of stimulator cells to induce IL2 production and their immunogenicity. Thus, the helper activity present in crude MLC supernatants [14, 15] appears to reside in IL2. It will be shown that at a later stage of a primary response (day 5–6), the CTL generated are extremely sensitive to IL2, and do not remain active when this factor is removed. This is similar to the effect of IL2 (TCGF) removal on long-term cultures of CTL [2, 17].

MATERIALS AND METHODS

Animals and Tumor Cell Lines

Breeding stock for CBA/CaJ (H-2k), CBA/J (H-2k), DBA/2J (H-2d), and (CBA/CaJxDBA/2J)F1 (H-2k/d) mice was obtained from Jackson Laboratories and maintained at the University of Alberta Health Sciences Animal Center. P815 (H-2d mastocytoma) and EL 4 (H-2b erythroleukemia) cells were maintained by passage in cell culture.

Generation and Measurement of CTL and MLR

Mouse thymic or splenic lymphocytes were cultured in 0.2 ml of RPMI 1640 medium containing 2-Me in V-bottom microwell trays (see [8]). Gamma irradiation of stimulator cells was at 2,500 rad, and UV irradiation was at 11 cm distance from a 15 watt germicidal lamp (intensity 1,600 microwatt/cm^2) for the times indicated. To assay for CTL, target cells were labeled with ^{51}Cr (New England Nuclear). Specific release of the label (F) was converted to a quantitative parameter similar to the alpha parameter defined by Miller and Dunkley [18]: $F = 1 - \exp(-N \cdot KA \cdot t)$, where N is the number of cells represented in a given well, t is the assay time in hours, and KA is "killing activity" [8]. Thymidine uptake into DNA was measured as described earlier [7].

Anti-PPD Response

Popliteal lymph node cells were obtained from CBA/CaJ mice primed to Mycobacterium in complete Freund's adjuvant by immunization in the hind footpads. They were cultured in RPMI 1640 lacking 2-Me, as described elsewhere [19]. Adherent cells were removed by passage over nylon wool columns. Gamma irradiated peritoneal cells from unstimulated mice were used as the source of macrophages [19].

Preparation and Assay of IL2 (Costimulator)

IL2 (fraction 4) was derived from Con A-stimulated mouse spleen cells, cultured in serum-free medium for 18–24 hours, and purified by ammonium sulfate precipitation, gel filtration chromatography and chromatography on DEAE Sephacel [8]. Various inbred mouse strains, as well as Swiss (outbred) mice, were used. The standard assay was the thymocyte proliferation assay in the presence of Con A [7]. DNA synthesis in these assays was measured by incorporation of either ^{3}H-thymidine or ^{125}I-UdR. Radioactive IUdR was obtained from the Radiopharmacy Center, Edmonton, at 1,850 Ci/mmole, and added to about 100,000 cpm/well in the assay. The incorporation of ^{125}I was about one-third as high, as a fraction of total radioactivity, as seen with ^{3}H, in identical cultures. The two radioactive precursors showed entirely parallel uptake in various experimental situations. The use of radioactive iodine offers the advantages of easier handling and analysis by gamma counting.

RESULTS

UV Irradiation of Stimulator Allogeneic Cells Inhibits CTL and Proliferation Responses and Also IL2 Induction

A number of alterations of stimulating alloantigenic cells can reduce their immunogenicity [11, 12]. The loss of CTL response after UV irradiation, and its restoration by partially purified IL2, is illustrated in Table I. Addition of IL2 to a normal response increases the level of CTL activity about 7–8-fold. UV irradiation reduces the response below detectability (more than 25-fold reduction), and addition of fraction 4 IL2 restores this to the maximal level seen with normal cells plus IL2. In other experiments (not shown), the cytotoxicity generated was shown to be specific for the immunizing alloantigen (cf 8). Furthermore, UV irradiation also blocks the ability of Con A-stimulated cells to generate IL 2 (Table I).

The synthesis of DNA (MLR response) seen in a typical one-way stimulation was also sharply inhibited by UV irradiation of the stimulating cells (Fig. 1). Again, addition of IL2 to either the normal, or UV-irradiated one-way stimulation cultures gave rise to about the same, elevated (about three-fold above normal) level of DNA synthesis.

The loss of immunogenicity observed after UV irradiation was accompanied by a loss of IL2 production (Fig. 2). (From previous results [16] it is known that essentially all of the activity produced under these conditions is IL2.). Thus, by 96 hours of culture, there was a fairly high level of IL 2 in one-way MLCs, but when the stimulating cells were UV irradiated, very little IL2 was produced. A low but significant amount of IL2 was present after about 96 hours (Fig. 2). Thus, one can conclude that neither stimulator nor responder cells generated significant levels of IL2 during the first 4 days of allogeneic stimulation if the stimulating cells were UV-irradiated. This is consistant with the lack of helper activity in crude MLC supernatants generated by UV-treated stimulators [14, 15].

TABLE I. IL2 Restores Immunogenicity to UV-Treated Cells

Stimulator cells	KA × 10^8 −IL2	KA × 10^8 +IL2	IL2 generated
None	< 4	< 4	6
(CBA × DBA) F1	92	690	300
F1, 0.1 min UV	4	710	20
F1, 0.2 min UV	4	640	4
F1, 1.0 min UV	4	660	6
F1, 2.0 min UV	4	560	4

Stimulator spleen cells were exposed to a UV lamp as described in Materials and Methods. They were then tested for their ability to stimulate a CTL response by CBA/J spleen cells in the presence or absence of fraction 4 IL2. CTL were assayed on P815 cells. The UV-treated cells were also tested for IL2 production in response to Con A, as measured in the standard thymocyte proliferation assay.

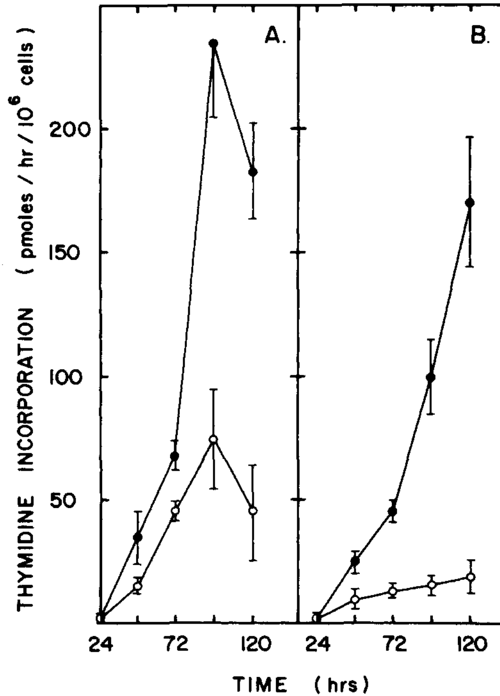

Fig. 1. Effects of UV irradiation of stimulating cells, and addition of IL2, on MLR. CBA/J spleen cells and gamma irradiated (2,500 rad) DBA/2J spleen cells, 1 × 10^6/ml each, were co-cultured in microtiter trays. Incorporation of tritiated thymidine into DNA was measured in 6 replicates by a 4.5-hour pulse at the times indicated (± standard deviation). A) (○) cells alone; (●) cells plus 6 U/ml fraction 4 IL2. B) (○) stimulator DBA/2J cells UV-irradiated for 2 minutes; (●) UV-irradiated DBA/2J cells plus 6 U/ml fraction 4 IL2.

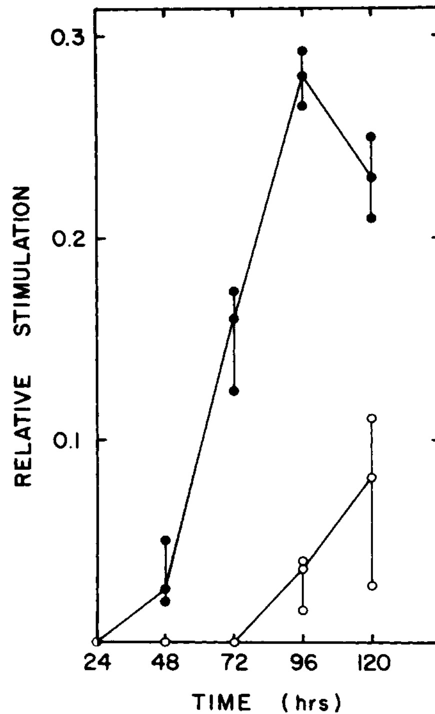

Fig. 2. Effect of UV irradiation on IL2 production in an MLR. CBA/J and gamma-irradiated DBA/2J spleen cells were co-cultured as in Figure 1, except that 16 × 100 mm glass tubes were used for the 1 ml cultures. (•) one-way MLR; (○) stimulator DBA/2J cells were also UV-irradiated (see Materials and Methods). Three separate cultures were set up and assayed at each time point. The assay for IL2 was the standard thymocyte proliferation with Con A, diluting each supernatant from the MLR 1:4, and establishing the maximal stimulation by adding a standard IL2 preparation to control cultures (maximum relative stimulation = 1.0). The assay points are thus all on the linear part of the assay scale (see [8]).

Furthermore, the helper activity in MLC supernatants can be accounted for by IL2. The data are also consistant with the hypothesis that IL2 is a normal mediator of induction of CTL.

There Is a Rapid Loss of CTL Activity in the Absence of IL2

In addition to the requirement for IL2 in the induction of CTL in cultures either deficient in helper cells [16, 20, 21] or containing UV-inactivated stimulator cells, there is a continuing requirement for IL2 later in the response, as shown in Table 2. When cells were washed free of IL2 and recultured, the CTL activity already generated was rapidly lost. Thus, the CTL activity on day 5 in the presence of IL2 was 17–18 $\times 10^{-8}$, but if IL2 was then removed, the activity was < 4 by the next day. As controls, removal of IL2 on the day of the assay had no effect, and adding back IL2 after its removal led to a continuing increase in the magnitude of the CTL response. These effector CTL behave

TABLE II. Effect of Removing IL2 From CTL-Generating Cultures During the First 5 Days

Day IL2 removed	Fresh IL2 added	KA × 10^8 (day of assay) 5	6	7
1	no	< 4	< 4	< 4
1	yes	< 4	32	42
2	no	< 4	< 4	< 4
2	yes	18	33	93
3	no	< 4	< 4	< 4
3	yes	17	74	116
4	no	< 4	< 4	< 4
4	yes	17	53	107
5	no		< 4	< 4
5	yes		37	93
7[a]	no			117
7	yes			139

Cultures (0.21 ml) contained 3 × 10^5 CBA/J thymocytes and 3 × 10^3 gamma-irradiated EL4 tumor cells as antigen. Fraction 4 IL2 was added at 4 units/ml to all cultures on day 0. Cultures were assayed for CTL on days 5, 6, or 7, using 2 × 10^4 ^{51}Cr-labeled target cells. IL2 was removed from cultures by centrifugation and aspiration. Where indicated, fresh IL2 was added back. KA × 10^8 for cultures containing responder cells only, and for responder cells plus IL2 were always < 4.
[a]Cells were washed and resuspended in fresh medium 0.5 hr before addition of target cells.

like long-lived T cell lines, which require IL2 for survival. Our conclusion, that CTL responses depend on IL2 being continually present, disagrees with that of Wagner et al [15] (see Discussion).

IL2 Is Adsorbed by Activated T Cells

Exogenous IL2 is relatively stable in tissue culture in the presence of nonresponding cells. Figure 3 shows that exogenous IL2 survived 4 days of culture with thymocytes, but if the thymocytes were simultaneously exposed to the "first signal" Con A, they actively removed IL2 from the medium. In this experiment, it was also shown that the thymocytes responded by proliferation in the presence of Con A plus IL2, as demonstrated elsewhere [1]. The IL2 activity remaining in the supernatant was assayed, with similar results, in both the thymocyte proliferation response (not shown) and the ability to confer responsiveness to thymocyte precursors for CTL in an allogeneic response, two assays thought to measure the same entity [5]. Similar results have been obtained by others for unpurified human TCGF [22], the putative IL2 activity in crude Con A supernatants of mouse cell cultures [23], and mouse TCGF acting on continuous T cell lines [2].

IL2 Replaces the Macrophage Requirement in a Secondary Proliferative Response Only Poorly

IL2 can apparently replace macrophages in cellular immune responses against cellular antigens [8, 24]. However, in the secondary proliferative response to a soluble antigen such as PPD, macrophages were only poorly replaced by IL2, as shown in

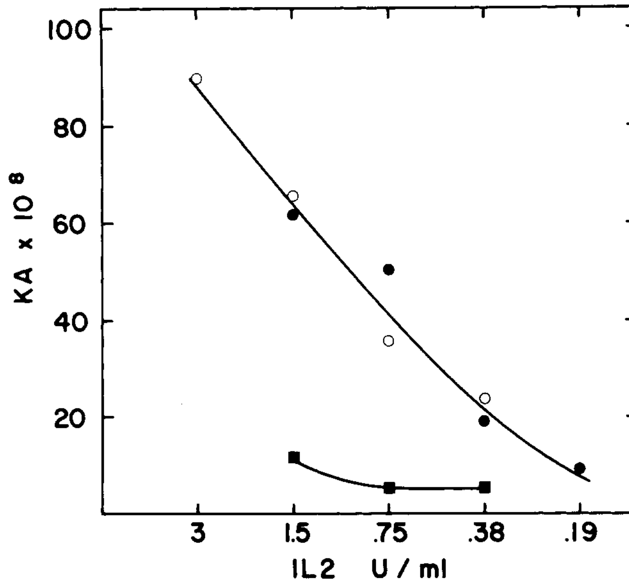

Fig. 3. Adsorption of IL2 by activated thymocytes. CBA/J thymocytes (2.5×10^6/ml) were cultured for 4 days with or without Con A (3 μg/ml) in the presence, initiallly, of 6 U/ml IL2 (fraction 4). After this incubation, the cells were removed by centrifugation and the supernatants were assayed for IL2, both in the thymocyte proliferation assay with Con A (data not shown) and in the CTL response of fresh CBA/J thymocytes to allogeneic C57B1/6 spleen cells (responder and stimulator cells at 1.25×10^6/ml). Cytotoxicity was measured on day 5 using ^{51}Cr-labeled EL4 target cells. The abscissa is in units of IL2/ml which would be present in the CTL-generating culture if there were no depletion during the first incubation. (○) IL2 standard (not exposed to thymocytes); (●) IL2 added to thymocytes cultured without Con A; (■) IL2 activity in cultures of thymocytes with Con A. The CTL response in the absence of IL2 was 7×10^{-8}.

Table III. In this experiment restoration of DNA synthesis by sensitized, A cell-depleted lymph node T cells was only about 14% as good when IL2 was added as when macrophages were added back. This would be expected if macrophages, in addition to providing the soluble mediator IL1 when properly stimulated, also have an antigen presentation function for soluble (as distinct from cellular) antigens (see Discussion).

DISCUSSION

The lympokine IL2 has several properties required of an essential product of helper T cells. In the first place, it is a product of T cells bearing the Ly markers of helper cells. It is generated only after stimulation with mitogen or antigen, and this requires participation by adherent cells, probably macrophages [8]. The A cell-activated T cell interaction is apparently at least partly I-A mediated [25]. A number of alterations known to reduce the immunogenicity of stimulator cells in cell-mediated immunity, including glutaraldehyde fixation [10] and UV irradiation [11, 12], are reversed by adding partially purified IL2 [16]; Table I). All of these observations argue, indirectly, for the participation of IL2 in normal CTL responses, at least in vitro.

TABLE III. Fraction 4 IL2 Only Partially Replaces the Requirement for Macrophages in a Secondary Proliferative Response

Responding cells	Additions	CPM	
		No PPD	+ PPD
Unfractionated	none	958	72,811
Nylon wool	none	81	1,810
Purified	IL2		
once	3 U/ml	6,825	33,820
	1.5 U/ml	2,252	21,829
	0.75 U/ml	1,781	12,282
	0.38 U/ml	353	7,294
	peritoneal cells		
	10^3	66	17,730
	3×10^3	112	54,502
	10^4	430	111,829
	3×10^4	1,679	131,664
	10^5	6,804	30,061
Nylon wool	none	465	1,891
Purified	IL2		
twice	3 U/ml	3,862	14,837
	1.5 U/ml	2,226	14,210
	0.75 U/ml	898	4,843
	peritoneal cells		
	10^3	272	8,874
	3×10^3	468	22,331
	10^4	207	69,567
	3×10^4	791	109,479
	10^5	7,539	55,015

Lymph node cells (2×10^5) from CBA/CaJ mice primed with Mycobacterium were enriched for T cells and depleted of macrophages by passage over nylon wool once or twice. The response was measured as the amount of ^{3}H-thymidine incorporated into DNA in the 24-hr prior to harvesting the cells at 120 hours. The results are expressed as total cpm incorporated. IL2 fraction 4 (Swiss) or irradiated peritoneal cells (40% macrophages) were added to cultures in the presence or absence of PPD (100 mg/ml).

The present work further supports the role of IL2 in CMI. Thus, the conversion of immunogenicity to mere antigenicity by UV irradiation of stimulator cells is accompanied by a failure of these cells to elicit IL2 production by the responding population. Partially purified (fraction 4) IL2 added to such cultures leads to strong CTL responses. Because even at the level of fraction 4 purity IL2 contains a small but significant level of immune interferon activity, it is not possible to discount the role of interferon in stimulation. However, it is now known that IL2 itself induces interferon production by T cells [26]. It is therefore impossible, except by specifically neutralizing interferon, to distinguish between IL2, which induces interferon and restores immunogenicity, and interferon itself. In either case, IL2 behaves like a necessary component of inter-leukocyte signalling during the generation of CMI.

The antigen-nonspecificity of IL2 is not a strong argument against its participation in antigen-specific T cell responses. A model of cell-cell interaction in which helper cells, macrophages, and precursors for CTL are associated, plus the known ability of activated T cells to adsorb IL2 ([2, 22, 23] Fig. 3), could insure that the nonspecific factor is induced, and acts, within the activating complex of cells. Such a model has been described more fully elsewhere [27]. Others [14, 15] have demonstrated that an antigen-nonspecific helper factor for CTL responses can be produced by antigen-specific T helper cells. Since we have shown elsewhere [7, 16] that all of the helper activity co-purifies with IL2, it seems likely that the helper activity generated from the antigen-specific T helper cells seen by others is in fact IL2. In summary, IL2 behaves like a nonspecific but essential second signal for cell mediated immune responses (cytotoxicity).

Although it is well known that IL2 is constantly required for the proliferation of long-term CTL lines [2, 17], there is conflicting evidence on its requirement by day 4 or 5 to maintain CTL activity [15, 28]. In Table II thymocyte precursors were used because they have a relative paucity of T helper cells [21]. They were rendered responsive by purified IL2. When this factor was removed, their response failed to expand in the 24-hour period between days 5 and 6; indeed, it actually declined to immeasurably low values. These data, together with the demonstration that there is no induction of CTL activity until both alloantigen and IL2 are present [8], show that in this type of response, IL2, or the resulting interferon activity, functions both early (in induction) and later (during clonal expansion and/or differentiation to cytotoxicity). Others [15] have concluded that IL2 is not important after about 48 hours of a 5-day CTL response. However, those experiments were performed using irradiated splenic stimulators, and it may be supposed that these are able to generate endogenous sources of help during the 48-hour culture period. In the results of Figure 2, for example, there was some induction of IL2 by 96 hours using UV-irradiated spleen cells as stimulators. Our experiments (Table II), similar to those of Baker et al [28], used tumor stimulator cells and demonstrated an unequivocal requirement for IL2 throughout the 6-day period of CTL generation. These tumor cells are unable to produce IL2 or any other form of help (unpublished observations). Again, these data indicate that helper effects present in Con A supernatants [28] are due to IL2.

That soluble products cannot replace macrophages or other adherent cells in all types of cellular responses is not surprising [19]. In the case of cellular antigens, the requirement for macrophages may, at least in vitro, be replaced by soluble components IL1 (a macrophage product) or IL2 (which is produced only if either macrophages or IL1 are present [27, 29, 30]). However, with soluble antigens such as PPD (Table III), macrophages may play a crucial additional role in antigen processing and presentation. Background cellular antigens, including apparently minor histocompatibility antigens, require macrophage processing to induce delayed type hypersensitivity, whereas major histocompatibility antigens do not [31]. In the experiments of Table III, thorough removal of adherent cells reduced the ability of IL2 to restore the proliferative response to PPD when compared to either normal cells or to depleted cells complemented with peritoneal macrophages.

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