REGULATION OF CELL-MEDIATED CYTOTOXICITY

I. Augmentation of Cell-Mediated Cytotoxicity Induced by Radiation*

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During the immune response to an allograft, lymphoid cells become capable of inducing specific in vitro lysis of target cells of donor's genotype. In some experimental situations, cell-mediated cytotoxicity (CMC)[1] was shown to be due to θ-bearing thymus-dependent cytotoxic lymphocytes (CL) (1, 2), while in other cases different cells may probably become cytotoxic (3, 4). Although the factors which regulate CMC are poorly understood, several pieces of evidence suggest that mechanisms of positive synergism of more than one cell type (5, 6), as well as suppressor mechanisms (7), operate in cell-mediated immune responses to histocompatibility antigens.

The experiments described here were prompted by the observation that the injection of allogeneic spleen cells into irradiated mice induced a stronger CMC than in nonirradiated ones. The results demonstrate that the precursors of the cytotoxic lymphocytes (P-CL) are relatively radio-resistant and can be triggered into a cytotoxic state by allogeneic or semiallogeneic lymphoid cells. The response of these cells appear to be regulated by radiosensitive thymus-dependent cells which, under the experimental conditions used for these experiments, acted as suppressor cells.

Materials and Methods

Animals.—Inbred, 7–9 wk old mice of both sexes of the strains A/J ($H\text{-}2^a$) and C57BL/6J ($H\text{-}2^b$) and their hybrids of the first generation ($B6AF_1$), obtained from the Jackson Laboratories, Bar Harbor, Main, were used for the CMC experiments. Males of the strains C3H/HeJ and AKR/J were used for the production of anti-θ serum.

Antisera.—The C57-anti-A and the A-anti-C57 sera were produced with six intraperitoneal injections of 1×10^7 $B6AF_1$ spleen cells, 4 wk apart, into C57BL/6 and A mice, respectively. The cytotoxicity of these antisera was measured using ^{51}Cr-labeled lymph node cells as targets in the presence of normal guinea pig serum used as a source of complement, following the procedure described in detail elsewhere (8). These antisera were used for the selective elimination of cells of a given genotype (A or C57BL/6), respectively) in a concentration calculated

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[1] *Abbreviations used in this paper:* CMC, cell-mediated cytotoxicity; GVH, graft-vs.-host; CL, cytotoxic lymphocytes; P-CL, precursors of the cytotoxic lymphocytes; SaI, Sarcoma I.

from the cytotoxic curve to represent approximately a four-fold excess of the amount of antiserum required for 100% lysis. Complement was also used in a fourfold excess.

The anti-θ-C3H serum was produced in AKR mice with six weekly intraperitoneal injections of 1×10^7 C3H thymus cells. The mice were bled 8 days after the last injection, the serum from 50 animals was pooled, heated at 56°C for 30 min, and stored at −20°C. This serum had a cytotoxic titer of 1:640 when tested with C3H and C57BL/6 thymus cells, was not cytotoxic to bone marrow cells of the same strains or to AKR thymus cells, and its cytotoxicity to C3H thymocytes was abolished by absorption with C3H or C57BL/6 brain but not with AKR brain.

For the selective elimination of T cells, spleen cells (2×10^7/ml) were incubated with the anti-θ-C3H serum diluted 1:2 in Hanks' balanced salt solution (Grand Island Biological Co., Grand Island, N. Y.) buffered at pH 7.2 with 0.01 M *N*-2-hydroethyl-piperazine-*N'*-2-ethanesulfonic acid (Hepes, Calbiochem., San Diego, Calif.) for 30 min at 4°C, washed once with the above solution, resuspended in agarose-absorbed (9) normal guinea pig serum diluted 1:5 and incubated at 37°C for 30 mins. The cells were washed twice before their intravenous injection into syngeneic mice.

Irradiation and Spleen Cell Injection.—Mice were subjected to whole body gamma irradiation using a ^{66}Co source. The source to midbody line distance was 100 cm; the dose rate was 60 rads/min. The spleen cells were injected 16 h after irradiation via the intravenous route.

Preparation of Spleen Cells.—The spleens were cut into pieces and teased with two needles in a tissue culture medium composed of RPMI 1640 medium (GIBCO) buffered at pH 7.2 with 0.04 M Hepes and supplemented with 10% fetal calf serum (GIBCO), 100 μg streptomycin and 100 U penicillin (Difco Laboratories, Detroit, Mich.) per ml. The cell suspension was filtered through a 200 mesh stainless steel screen and the cells were washed twice before their use in the CMC assay. For intravenous injection the cells were prepared as above in Hanks' solution (GIBCO), washed three times, and finally resuspended at the concentration of 2×10^8 cells/ml.

Target Cells.—The tumors (both obtained from the Jackson Laboratories) B16 melanoma, transplantable in C57BL/6 mice, and Sarcoma I (SaI), transplantable in A mice, were used as the sources of target cells for the CMC assay. B16 cells were cultured before their use as target cells, SaI was grown as an ascites tumor. The methods for the preparation, culture, and ^{51}Cr-labeling of these cells were described elsewhere (10).

CMC Assay.—This test was described elsewhere in detail (8). Briefly, it consisted of mixing in the wells of tissue culture plates (Microtest II, Falcon Plastics, Div. of Bioquest, Los Angeles, Calif.) 0.2 ml of a spleen cell suspension (2×10^6 cells) with 0.05 ml of a suspension of target cells (2×10^4 cells) labeled with ^{51}Cr and incubating the plates at 37°C for 16 h. The plates were then centrifuged (250 *g* for 8 min) and 0.1 ml of the cell-free supernate was collected for the measurement of the ^{51}Cr released. The per cent corrected lysis was calculated according to the expression (E-C)/(T-C) × 100, where E = cpm in the supernate of the experimental well, C = cpm in the supernate of the control wells containing normal spleen cells, and T = cpm in the supernate of wells in which 100% target cell lysis had been induced by freezing and thawing three times.

RESULTS

Cytotoxicity of the Spleen Cells after Allografting with Skin or Lymphoid Cells.—In Table I, the cytotoxic effect of spleen cells of A mice which had received 10 days earlier an allograft of skin from the tail of C57BL/6 donors is compared with that of spleen cells of animals of the same strain 8 days after an intravenous injection of C57BL/6 spleen cells. Skin grafts induced strong CMC, while the injection of allogeneic spleen cells did not have a similar

effect. By contrast, the irradiated recipients of allogeneic spleen cells developed marked CMC. Thus, irradiation, which would be expected to suppress cell-mediated immunity, actually enhanced the CMC response to a treatment which was ineffective in normal mice.

Kinetics of the Development of CMC in Mice Injected with Semiallogeneic Spleen Cells.—For the study of the kinetics of the formation of killer cells in the spleens of irradiated and nonirradiated mice, semiallogeneic F_1 hybrid spleen cells were injected to avoid any graft-vs.-host (GVH) reaction. Both in the combination $B6AF_1$ to C57BL/6 (Fig. 1) and in the combination $B6AF_1$ to A (Fig. 2), irradiation in the two doses of 500 and 700 rads affected the

TABLE I

Cytotoxicity of Spleen Cells of Mice Immunized with Skin Allografts or with the Injection of Allogeneic Spleen Cells and Effect of Irradiation

Irradiation	Immunization*	Lysis‡
rads		
—	A skin	1.5 ± 3.3
—	C57 skin	78.4 ± 2.7
—	1×10^8 A spleen cells	−2.2 ± 6.4
—	1×10^7 C57 spleen cells	7.8 ± 4.5
—	1×10^8 C57 spleen cells	3.5 ± 4.1
500	1×10^8 C57 spleen cells	56.4 ± 7.2

* Mice of strain A were orthotopically grafted with syngeneic (A) or allogeneic (C57BL/6) skin or injected intravenously with syngeneic or allogeneic spleen cells. The skin grafted animals were sacrificed on day 10 and those injected with spleen cells on day 8 after the immunization.

‡ Mean percent corrected lysis of B16 target cells ± SE in groups of five animals.

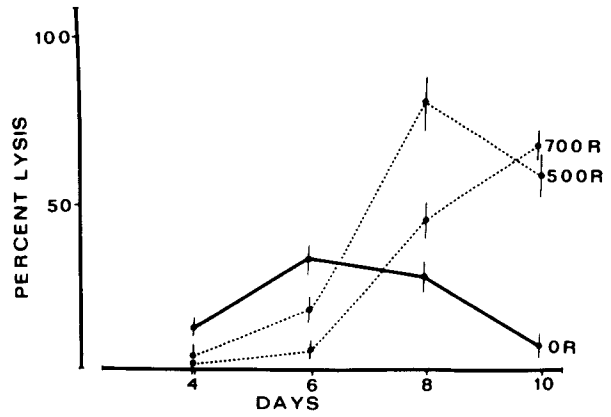

FIG. 1. Effect of different doses of radiation on the kinetics of the appearance of cytotoxic cells in the spleens of C57BL/6 mice injected intravenously with 1×10^8 $B6AF_1$ spleen cells. Each point represents the mean lysis of SaI target cells in a group of three mice.

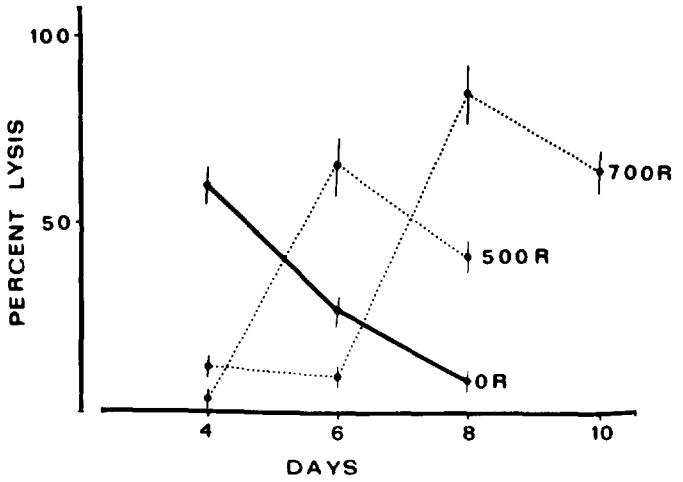

FIG. 2. Effect of different doses of radiation on the kinetics of the appearance of cytotoxic cells in the spleens of A mice injected intravenously with 1×10^8 $B6AF_1$ spleen cells. Each point represents the mean lysis of SaI target cells in a group of three mice.

kinetics of the development of CMC by inducing a delay in the appearance of the peak of maximum CMC as compared with the nonirradiated controls. In both combinations, the maximal lysis caused by the spleen cells of the irradiated mice was higher than the maximal lysis caused by the spleen cells of the nonirradiated controls. Similar experiments were performed with the injection of allogeneic spleen cells, i.e., C57BL/6 to A and A to C57BL/6. The results (not reported here) demonstrated a similar kinetics and comparable levels of maximal cytotoxicity as described in the animals receiving F_1 hybrid cells. Therefore, the increased cytotoxicity of spleen cells of irradiated mice 8 days after the injection of allogeneic spleen cells reported in Table I should be attributed in part to the different kinetics of the response and in part to a true augumentation of CMC.

Specificity of CMC and Role of Donor and Host Cells.—The results reported above failed to establish if CMC was specific for target cells of donor's genotype. This was studied with the use of target cells derived from the donor and host strains and at the same time the origin of the killer cells was established with the use of specific antisera cytotoxic either to the donor or to the host cells. Mice of strain C57BL/6 were irradiated (500 rads) and injected intravenously with A spleen cells. 8 days later the animals were killed and their spleen cells divided into portions which were either left untreated or incubated with A-anti-C57 serum (group 6 of Table II) or C57-anti-A serum (group 5) in the presence of fresh guinea pig serum. For control purposes, normal mouse serum with complement (group 3) and A-anti-C57 serum in the presence of heated (56°C for 30 min) guinea pig serum (group 4) were also used. The CMC assay, carried out with B16 (C57BL/6 genotype) and SaI (A genotype) target

TABLE II

Role of Donor and Host Cells in the Lysis of Target Cells of Donor's and Recipient's Genotype Induced by the Spleen Cells of Irradiated Mice Injected with Allogeneic Spleen Cells

Group	Irradiation (500 rads)	Treatment of spleen cells*	Lysis‡	
			B16 cells	SaI cells
1	−	—	6.2 ± 4.2	3.9 ± 2.2
2	+	—	18.0 ± 6.4	91.7 ± 3.2
3	+	NMS	14.8 ± 3.1	85.3 ± 1.4
4	+	A-anti-C57 + HC	13.1 ± 2.9	78.5 ± 5.3
5	+	C57-anti-A	−2.5 ± 1.3	80.6 ± 2.4
6	+	A-anti-C57	19.0 ± 5.6	7.6 ± 3.3

* A group of five C57BL/6 mice was irradiated and 1×10^8 A spleen cells were injected intravenously into each animal. 8 days later the animals were sacrificed and their spleen cells were pooled, divided into portions which were left untreated (group 2) or incubated for 45 min at 37°C with normal mouse serum (NMS) and guinea pig complement (group 3), with A-anti-C57 serum (diluted 1:10) and heated (56°C for 30 min) guinea pig serum (group 4), C57-anti-A serum (diluted 1:10) and guinea pig complement (group 5) or A-anti-C57 serum (diluted 1:10) and guinea pig complement (group 6). The nonirradiated controls (group 1) received 1×10^8 A spleen cells.

‡ Mean percent corrected lysis of three replicates ± SE of B16 (C57BL/6 genotype) or SaI (A genotype) target cells.

cells, demonstrated that the untreated spleen cells of irradiated hosts (group 2) induced marked lysis of SaI cells and only weak lysis of B16 cells. The treatment of these spleen cells with C57 anti-A serum (antidonor) abolished completely the lysis of B16 target cells without affecting the lysis of SaI cells, while the treatment with A-anti-C57 serum (antihost) abolished completely the lysis of SaI. This effect was complement-dependent and normal mouse serum and complement did not significantly affect lysis. These results demonstrate that some of the donor cells had survived 8 days after their injection and had become sensitized to host's antigens (GVH reaction) while host cells were reacting against the donor cells. Both the GVH and the host-vs.-graft reactions induced specific lysis of the respective targets. Thus, the CMC assay with target cells of donor's genotype measured a specific immune reaction of host's cells.

The results obtained in a similar experiment in which $B6AF_1$ spleen cells were injected into C57BL/6 recipients also demonstrated the specificity of the cytotoxic mechanism. In this case untreated spleen cells of the irradiated mice (group 2 of Table III) induced a marked lysis of SaI cells but no significant lysis of B16 cells, thus indicating that no GVH reaction had occurred, as expected, after the injection of F_1 hybrid cells. The lysis of SaI cells was abolished by the A anti-C57 serum (group 6) but was not affected by the same serum in the absence of complement activity (group 4) or by the C57-anti-A serum (group 5).

The results of Tables II and III clearly establish the specificity of cytotox-

TABLE III

Role of Donor and Host Cells in the Lysis of Target Cells of the Genotype of the Recipient and of the Other Parental Strain Induced by the Spleen Cells of Irradiated Mice Injected with F_1 Hybrid Spleen Cells

Group	Irradiation (500 rads)	Treatment of spleen cells*	Lysis‡	
			B16 cells	SaI cells
1	−	—	3.4 ± 2.3	6.1 ± 3.3
2	+	—	5.8 ± 1.9	62.5 ± 2.1
3	+	NMS	6.2 ± 0.9	57.4 ± 1.8
4	+	A-anti-C57 + HC	3.4 ± 1.7	61.1 ± 2.3
5	+	C57-anti-A	−1.4 ± 3.7	70.6 ± 0.5
6	+	A-anti-C57	0.5 ± 4.1	2.3 ± 2.2

* The experimental design described for the experiment reported in Table II was followed except that the mice received the injection of 1×10^8 $B6AF_1$ spleen cells.

‡ Mean percent corrected lysis of three replicates ± SE of B16 (C57BL/6 genotype) or SaI (A genotype) target cells.

icity and the host origin of the cytotoxic cells capable of lysing target cells of donor's genotype. Therefore, these must have derived from radioresistant precursors.

Suppression of CMC in Irradiated Mice with Lymphoid Cells.—Since irradiation eliminates from the lymphoid organs a large number of radiosensitive cells, it is conceivable that the elimination of such cells may be the reason for the phenomena of altered kinetics and increased CMC response reported above. To test this possibility, syngeneic lymphoid and hemopoietic cells obtained from different tissues were injected into irradiated mice immediately after the injection of semiallogeneic spleen cells. The results of an experiment in which mice of strain A received a dose of 8×10^7 syngeneic spleen, thymus, lymph node, or bone marrow cells are reported in Fig. 3. All these treatments inhibited the cytotoxicity of the recipients' spleen cells, thus suggesting that the syngeneic cell inocula may have supplied some cells endowed with the property of suppressing CMC. The experiment reported in Fig. 4 demonstrates the same effect induced by spleen and thymus cells in C57BL/6 mice. The measurement of the suppressor activity with different cell doses shows that the thymus cell population is about twice as active as the spleen cell population.

Time Required for the Suppressor Effect of Spleen Cells.—To study the mechanism of the suppression of CMC induced by the injection of syngeneic spleen cells in irradiated mice, these were administered at different time intervals after irradiation, i.e., at the same time or 1, 2, 4, and 7 days after the injection of semiallogeneic spleen cells which were always given 16 h after irradiation. The results reported in Table IV demonstrate that the suppressor effect was marked if the syngeneic spleen cells were injected 4 or more days before the CMC assay but was not observed if these cells were given 24 h before the test. Since this time is sufficient for the localization of the injected cells in the re-

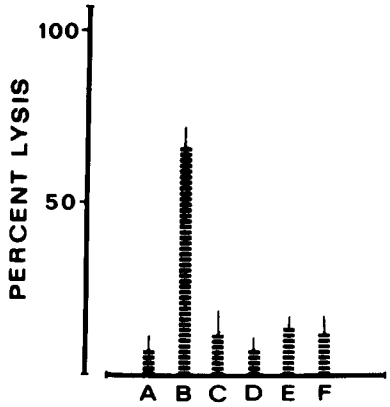

FIG. 3. Suppression of CMC by syngeneic cells. Groups of three mice of strain A received: 1×10^8 $B6AF_1$ spleen cells intravenously (A), 600 rads whole body irradiation and 1×10^8 $B6AF_1$ spleen cells (B), or irradiation, 1×10^8 $B6AF_1$ spleen cells and 8×10^7 syngeneic spleen cells (C), thymus cells (D), lymph node cells (E), or bone marrow cells (F).

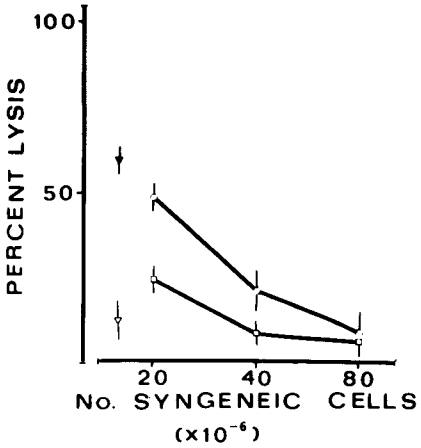

FIG. 4. Suppression of CMC by different doses of syngeneic spleen or thymus cells. Groups of three mice of strain C57BL/6 received 1×10^8 $B6AF_1$ spleen cells intravenously (▽), 500 rads whole body irradiation and 1×10^8 $B6AF_1$ spleen cells (▼), or irradiation, 1×10^8 $B6AF_1$ spleen cells and syngeneic spleen (○), or thymus cells (□) in different doses.

cipients' spleens (11), the suppressor cells probably acted before the appearance of CL by preventing the activation of their precursors, but probably had no inhibitory activity on active CL.

Thymus Dependence of Suppressor Cells.—Since both thymus and spleen cells had a suppressor effect, it was considered possible that this effect was due to a cellular moiety common to both populations. To test this possibility, spleen cells were treated with anti-θ serum and complement before their injec-

tion into syngeneic irradiated mice treated with semiallogeneic spleen cells. This treatment of spleen cells resulted in the abolition of the suppressor effect of spleen cells (Table V), thus indicating that the suppressor activity was due to T cells.

TABLE IV

Suppressor Effect on CMC of Syngeneic Spleen Cells Given at Different Intervals after the Injection of F_1 Hybrid Spleen Cells

Syngeneic cells given on*	Irradiation	Lysis‡
No syngeneic cells	—	6.4 ± 5.5
No syngeneic cells	+	38.7 ± 7.3
Day 1	+	9.2 ± 5.9
Day 2	+	11.4 ± 4.7
Day 4	+	6.3 ± 7.7
Day 7	+	37.5 ± 8.3

* Mice of strain C57 BL/6 received 500 rads of whole body radiation on day 0, 16 h later they were injected with 1×10^8 $B6AF_1$ spleen cells; 8×10^7 C57BL/6 spleen cells were given immediately after the $B6AF_1$ spleen cells (Day 1) or 1 (Day 2), 3 (Day 4) or 6 (Day 7) days later. The nonirradiated controls and one group of irradiated mice received only 1×10^8 $B6AF_1$ spleen cells. CMC was measured on day 8.

‡ Mean percent corrected lysis of SaI target cells ± SE in groups of three animals.

TABLE V

Sensitivity of Suppressor Cells to Anti-θ Serum

Irradiation (500 rads)	Treatment of syngeneic cells*	Lysis‡
—	No syngeneic cells given	3.3 ± 4.1
+	No syngeneic cells given	49.3 ± 3.3
+	No treatment	8.9 ± 5.1
+	NMS	11.5 ± 6.8
+	Anti-θ	42.5 ± 3.7

* C57BL/6 mice received 500 rads of whole body radiation, 16 h later they were injected with 1×10^8 $B6AF_1$ spleen cells immediately followed by 8×10^7 syngeneic spleen cells either untreated or treated with normal mouse serum and guinea pig complement (NMS) or with anti-θ serum and guinea pig complement (anti-θ). The nonirradiated controls and a group of irradiated mice received only 1×10^8 $B6AF_1$ spleen cells.

‡ Mean corrected percent lysis of SaI target cells ± SE of groups of three animals.

DISCUSSION

The results reported here provide evidence in favor of complex mechanisms of regulation of the immune responses to allografts. Among the factors which affect the response of the host, the nature of the transplanted tissue, and perhaps also the route of presentation, is of paramount importance. Thus, skin and tumor allografts induce the development of strong CMC, while the intravenous injection of allogeneic lymphoid cells was shown here to stimulate a

lower and short-lived CMC reaction. Moreover, irradiation induced a modified and somewhat increased response in the animals treated intravenously with allogeneic lymphoid cells, while the same treatment was immunosuppressive in tumor and skin allografted mice (unpublished results). This indicates that different mechanisms of regulation operate in these two situations and that radiation can be used to evidence such differences. While the clarification of these regulatory mechanisms must await further experimentation, two aspects clearly emerge from the present experiments, i.e., (*a*) the radioresistance of the P-CL and (*b*) the suppressor activity of radiosensitive θ-bearing cells.

The P-CL were shown to resist irradiation up to a dose of 700 rads. A more precise definition of the radiosensitivity of these cells was made difficult by the high mortality of mice induced by radiation doses higher than 700 rads, since the animals could not be protected with syngeneic hemopoietic cells without affecting at the same time the development of CMC. It would appear, however, that the radiation dose of 900 rads reduced drastically CMC in the recipients of semiallogeneic spleen cells (unpublished results). This indicates that the P-CL had only partial resistance to radiation and may be tentatively identified with the cells involved in the inactivation of allotransplanted hemopoietic stem cells (12) and with the sessile T cells described by Stobo et al. (13) which present similar partial resistance to radiation.

The augmentation of CMC induced by radiation in the recipients of allogeneic and semiallogeneic spleen cells should be attributed to the elimination of radiosensitive cells. The observation that doses of syngeneic spleen and thymus cells insufficient for a full reconstitution of the host were enough to suppress CMC in the irradiated mice, even if these cells were given 4 days after the injection of F_1 hybrid spleen cells, indicates that this phenomenon was not solely due to the creation of space for radioresistant cells in the hosts' spleens or to a "rebound" effect. This conclusion is further supported by the abolition of the suppressor effect after the selective elimination of T cells from the syngeneic spleen cell inoculum which indicates that the suppression of CMC results from a specific function of immunocompetent cells. Thus, the suppressor cells demonstrated in these experiments are probably of the same nature as the suppressor cells detected in other experimental models of cell-mediated immunity (7). However, it ought to be pointed out, that the expression suppressor cells is used here merely as a descriptive term to denote the effect observed and does not necessarily imply that suppression is the sole function of a distinct class of cells. While this may well be the case, the fact that nonirradiated mice treated with the intravenous injection of allogeneic and semiallogeneic spleen cells developed a rapid and transitory CMC response suggests that the suppression induced by syngeneic T cells may also be preceded by a short phase of stimulation of CMC. In this case, the suppression of CMC by radiosensitive T cells described here may be interpreted as due to an excess of cells which induced a rapid activation of P-CL and an equally

rapid decline of the response. Thus, it cannot be excluded that the same suppressor cells, under different experimental conditions, may stimulate CMC. This interpretation, which has the advantage of simplifying the mechanisms of regulation of CMC, is supported by observations in other experimental situations of short lived immune responses before the induction of suppressor mechanisms (14). Further studies are clearly indicated for the solution of these problems.

SUMMARY

Mice were treated with sublethal and midlethal doses of irradiation (500–700 rads) and injected intravenously with allogeneic or semiallogeneic F_1 hybrid spleen cells. The cytotoxicity developed by their spleen cells was measured with the lysis of ^{51}Cr-labeled target cells and was found to be stronger (although delayed in time) than the cytotoxic activity of spleen cells from nonirradiated mice. The injection of syngeneic thymus or spleen cells in the irradiated mice after their treatment with allogeneic spleen cells exerted a suppressor activity, i.e., reduced the level of cell-mediated cytotoxicity (CMC). The majority of effector cells involved in the modified CMC response of irradiated mice was shown to be of host origin and lysed specifically target cells of the same genotype as the donor. A small percentage of the cells obtained from the spleens of irradiated recipients of allogeneic spleen cells was composed of donor cells which lysed specifically target cells of the same genotype as the host. These results demonstrate that the precursors of the cytotoxic cells responsible for target cell lysis are relatively radioresistant and suggest that their response is regulated by radiosensitive thymus-dependent cells.

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