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Suppression of Adoptive Antituberculosis Immunity by Normal Recipient Animals

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Adoptive immunity is poorly expressed in normal syngeneic mice. This phenomenon was studied by using experimental antituberculosis immunity as a model system representing pure cell-mediated immunity. Expression of adoptive immunity was facilitated by pretreating recipients with sublethal ionizing radiation (500 rads) or high doses (200 mg/kg) of cyclophosphamide or by using adult thymectomized, lethally irradiated, bone-marrow-reconstituted (TXB) mice. Adult thymectomy was less effective, and a low dose of cyclophosphamide (20 mg/kg) was completely ineffective. The beneficial effect of sublethal irradiation was reduced over time; it persisted for 4 weeks and was absent after 8 weeks. Attempts to restore the suppressed state of normal mice to sublethally irradiated mice by using normal spleen or thymus cells did not succeed. Even in rats, which express adoptive antituberculosis immunity without immunosuppressive treatment, the use of sublethally irradiated or TXB recipients potentiated adoptive immunity. It was concluded that suppression of adoptive immunization in normal recipient mice is mediated predominantly, if not exclusively, by T lymphocytes that are sensitive to a number of immunosuppressive agents. The suppressor cells are long-lived and can be regenerated from precursors that are resistant to 500 but not to 900 rads of ionizing radiation.

It has long been known that adoptive immunity is not expressed well by recipients of sensitized lymphocytes unless the recipients are first exposed to sublethal doses of total-body ionizing radiation (13, 25). Such irradiation has been found to promote the expression of humoral (7, 8, 13, 25) and cell-mediated immunity (2).

The successful transfer of adoptive antituberculosis immunity in mice was first reported in 1975 (16). It was found that successful transfer could not be achieved in normal syngeneic recipients, but only in those that had been exposed to total-body ionizing radiation. As little as 250 rads of X rays facilitated the expression of adoptive immunity, but immunity was maximal after 500 rads, a convenient dose because it is sublethal. Since then, 500 rads of X or γ radiation has been used as a standard dose in studies of adoptive antituberculosis immunity in mice (17–19). However, a different situation prevails in rats, in which such immunity is expressed well in normal, unirradiated recipients (21).

The mechanism by which the expression of sensitized lymphocytes is suppressed in normal recipients remains unknown. An early hypothesis was that irradiation reduced the size of the lymphocyte pool of the recipient, providing more physical space within which sensitized donor cell clones could expand (25). Alternatively, it was suggested that depletion of the lymphocyte pool of recipient mice might facilitate a higher probability of random contact between the transferred sensitized lymphocytes and the appropriate antigen (8). More recent developments in immunology suggest the possibility that ionizing radiation deletes some cell population that regulates the expression of immunity in some positive fashion (22, 26, 27). The purpose of this study was to test these hypotheses.

MATERIALS AND METHODS

Animals. Female B6D2 F_1 [(C57BL/6J × DBA/2J)F_1] mice were obtained from The Jackson Laboratories, Bar Harbor, Maine, and used for experiments at 6 to 8 weeks of age. Female LBN [(Lewis × BN)F_1] rats were obtained from the Trudeau Institute, Saranac Lake, N.Y., and used at the same age.

Irradiation. Animals were exposed to total-body ionizing radiation from a ^{137}Cs source delivering 123 rads/min.

Thymectomy. Six-week old animals were anesthetized with ether and thymectomized (TX mice) by the cervical route with the aspiration technique (12). At 1 week after thymectomy, some animals were lethally irradiated by exposure to 900 rads. Later the same day

these animals were given 2.5×10^6 syngeneic bone marrow cells intravenously (i.v.). Such animals are designated TXB (thymectomized, lethally irradiated, bone marrow reconstituted) mice and were not used in experiments until at least 8 weeks after the bone marrow transfer.

Cortisone. Cortisone acetate was given to recipient mice as a single 5-mg subcutaneous injection 1 week before cell transfer.

CY. Cyclophosphamide (CY) was freshly dissolved in sterile saline and injected i.v. into recipient mice at a dose of either 20 or 200 mg/kg 1 or 2 days before cell transfer.

Mycobacteria. Living cells of *Mycobacterium bovis* BCG, Pasteur strain (TMC 1011), were grown in Proskauer and Beck medium containing 2% glycerol and 0.1% Tween 80. *Mycobacterium tuberculosis* R1Rv (TMC 205) was grown in Middlebrook 7H-9 medium (Difco Laboratories, Detroit, Mich.). Both cultures were distributed into vials and stored at −70°C.

Lymphoid cells. Mouse thymus glands and spleens were dissociated by compression through stainless-steel sieves. Thymus cells were washed twice with Dulbecco phosphate-buffered saline (PBS). Spleen cells were washed once in PBS, once in ammonium chloride-Tris buffer to lyse erythrocytes (5), and once more in PBS. Thoracic duct lymphocytes were obtained from rats after cannulation of the thoracic duct and overnight drainage. The cells were collected into PBS containing 10 U of heparin per ml and washed twice in PBS. Total and viable (determined by trypan blue exclusion) leukocyte counts were made on all cell populations, after which the cells were appropriately diluted in PBS to the desired concentration and injected i.v.

Immunization. The BCG culture was diluted in saline to contain 2.5×10^7 viable units per ml, and then 0.04 ml was injected into one hind footpad of each mouse and 0.1 ml was injected into both hind footpads of each rat. The inocula were 10^6 and 5×10^6 viable bacilli for mice and rats, respectively.

Challenge. Strain R1Rv cells were diluted in saline to a concentration of 5×10^5 viable bacilli per ml, and 0.2 ml (10^5 organisms) was injected into the tail veins of the mice and rats approximately 1 h before cell transfer. The challenged animals were killed 14 days later, and each spleen was removed, separately homogenized, and quantitatively plated on Middlebrook 7H-10 agar (Difco Laboratories). After these plates were incubated for 21 days at 37°C, colonies of strain R1Rv cells were counted, and the number of viable bacilli per spleen was estimated. The geometric mean viable organism count for each group of animals was then calculated.

Statistics. Viable counts were expressed to $\log_{10}$, and these data were evaluated by analysis of variance. Comparison between means was then done by the Newman-Keuls modification of the Q test. Unless otherwise stated, there were five animals per group.

RESULTS

Facilitation of expression of adoptive antituberculosis immunity. Groups of mice were treated in one of the following ways: no treatment, adult thymectomy (TX mice), adult thymectomy-lethal irradiation-bone marrow reconstitution (TXB mice), sublethal (500 rads) irradiation, or cortisone (5 mg subcutaneously). Half of each treatment group was then given 2×10^7 spleen cells from BCG-immunized mice. The cell recipients and their corresponding controls were then challenged with strain R1Rv. The interval between thymectomy and admission to the experiment was 4 months. Considering first the groups that received no cells, the number of strain R1Rv cells per spleen was similar except in mice exposed to 500 rads, in which significantly higher counts ($P < 0.05$) were observed than for any other group (Fig. 1). The increased growth of strain R1Rv cells in the spleens of mice exposed to 500 rads compared with growth in untreated recipients was a consistent feature of the entire series of experiments, but the differences were not always statistically significant. The resistance conferred by sensitized spleen cells was statistically significant ($P < 0.01$) in every type of recipient except the untreated mice, confirming earlier studies (16). However, the greatest antituberculosis immunity was seen in irradiated and TXB mice, which expressed significantly greater resistance ($P < 0.01$) than the cortisone-treated or TX mice. It was inferred that a variety of immunosuppressive maneuvers facilitated the expression of adoptive antituberculosis immunity, the most efficient of which were sublethal irradiation and TXB treatment.

Duration of effect of sublethal irradiation. Groups of recipient mice were exposed to 500 rads either at the time of (week 0) or 1, 2, 3, or 4 weeks before the transfer of sensitized spleen cells and challenge with strain R1Rv (Fig. 2). Among the mice that received no cells, significantly higher counts of strain R1Rv cells compared with those in unirradiated controls were obtained in mice irradiated at week 0 ($P < 0.01$). High levels of adoptive immunity ($P < 0.01$) were observed in all the irradiated recipients of sensitized cells, but not in the unirradiated recip-

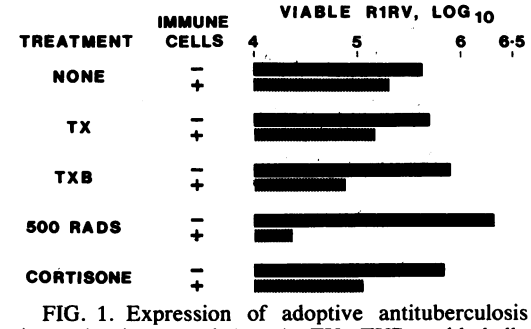

FIG. 1. Expression of adoptive antituberculosis immunity in normal (none), TX, TXB, sublethally irradiated (500 rads), and cortisone-treated mice. Mice were given no (solid bars) or 2×10^7 (broken bars) sensitized spleen cells.

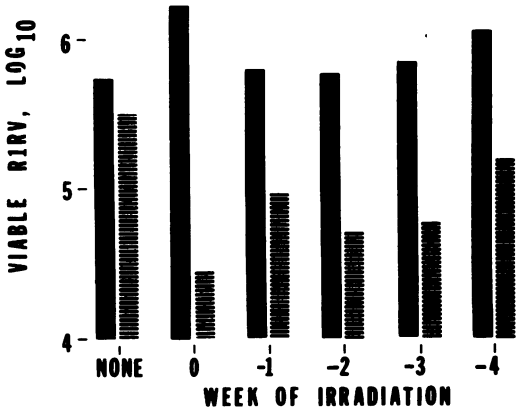

FIG. 2. Effect of the time (number of weeks before challenge) at which recipients were irradiated on the expression of adoptive immunity. Mice were given no (solid bars) or 2×10^7 (broken bars) sensitized spleen cells.

ients. The magnitude of adoptive immunity was similar to mice irradiated at week 1, 2, 3, or 4 before challenge, but significantly greater protection ($P < 0.01$) was expressed in mice irradiated at week 0. This maximal immunity appeared to be due in part to lower absolute counts of strain R1Rv organisms in cell-recipient mice irradiated at week 0, and in part to higher absolute counts in the corresponding controls that received no cells. It is pertinent that the immunopotentiating effect of irradiation prevailed for the full 2 weeks of the assay for adoptive antituberculosis immunity.

Effect of combining sublethal irradiation and T lymphocyte depletion. An earlier experiment indicated that T lymphocyte depletion alone might permit the full expression of antituberculosis adoptive immunity (Fig. 1). This hypothesis was tested by measuring the effect of sublethal irradiation on the expression of adoptive immunity in mice that had previously been depleted of T lymphocytes.

In the first experiment, TXB and normal mice were used as recipients, half having been exposed to 500 rads immediately before challenge with strain R1Rv. One group of mice were given 10^6 spleen cells from BCG-immunized donors. The interval between thymectomy and admission to the experiment was 9 weeks. In this experiment, increased growth of strain R1Rv ($P < 0.01$) was seen in intact irradiated control mice and in control TXB mice, whether irradiated or not (Table 1). The variation in counts between these three groups was not statistically significant. These same groups expressed very high levels of protection that differed significantly from those seen in normal unirradiated mice ($P < 0.01$), but not among themselves.

The same experimental design was used to evaluate TX mice (Table 1). In this experiment, in distinction to that shown in Fig. 1, the TX mice were used only 4 weeks after thymectomy. Perhaps for this reason neither the unirradiated intact nor the unirradiated TX recipients of sensitized spleen cells expressed adoptive immunity, but high levels of protection were expressed by both groups after sublethal irradiation ($P < 0.01$).

Use of sublethal irradiation to eliminate T lymphocytes from TX mice. The previous experiments revealed that adoptive immunity was not expressed as fully in TX mice as in TXB and sublethally irradiated mice. Moreover, adoptive immunity was not expressed at all in TX mice until several months after thymectomy. It was thought that the combination of adult thymectomy with sublethal irradiation, by reducing the number of residual T lymphocytes, might facilitate the expression of adoptive immunity on a long-term basis.

Accordingly, prospective recipients were either thymectomized or untreated 9 weeks before challenge, and groups of TX and normal recipients were exposed to 500 rads either 1 or 9 weeks (week 0) later. At week 0, all the mice were challenged with strain R1Rv, and 10^6 spleen cells from BCG-immunized mice were injected i.v. into half the animals. Viable counts of strain R1Rv cells were made from the spleens 2 weeks later (Table 2).

Adoptive immunity was not expressed in intact mice that had not been irradiated at all or irradiated 8 weeks before challenge (Table 2). By contrast, adoptive immunity was expressed optimally in mice irradiated at week 0 regardless of whether or not they had also been thymecto-

TABLE 1. Combined effects of T cell depletion and sublethal irradiation on the expression of adoptive immunity

Mouse group	Irradiation (rads)	Log_{10} viable bacilli per spleen in mice receiving:		Immunity[b]	P
		No cells	10^6 cells[a]		
Normal	0	5.5166	5.3423	0.1743	NS[c]
Normal	500	5.9568	4.6626	1.2942	<0.01
TXB	0	6.1082	4.9145	1.1937	<0.01
TXB	500	6.1754	4.7354	1.4400	<0.01
Normal	0	5.3078	5.4363	−0.1285	NS
Normal	500	5.4954	4.6386	0.8568	<0.01
TX	0	5.4378	5.3276	0.1102	NS
TX	500	5.4000	4.6590	0.7410	<0.01

[a] Obtained from BCG-immunized donors.
[b] Count for mice receiving cells subtracted from count for mice receiving no cells.
[c] NS, Not significant.

TABLE 2. Recovery of suppressor status of TX mice after sublethal irradiation[a]

Thymectomy	Irradiation (rads)		Log_{10} viable bacilli per spleen in mice receiving:		Immunity[c]	P
	Week −8	Week 0	No cells	10^6 cells[b]		
No	0	0	5.6881	5.6155	0.0726	NS[d]
No	0	500	6.2738	5.2186	1.0552	<0.01
Yes	0	500	6.1929	4.9508	1.2421	<0.01
No	500	0	5.6099	5.4865	0.1234	NS
Yes	500	0	6.2251	5.7064	0.5187	<0.01

[a] Thymectomy was performed 9 weeks before challenge. Mice were irradiated either 8 weeks (week −8) or immediately before (week 0) challenge.
[b] Obtained from BCG-immunized donors.
[c] See Table 1, footnote *b*.
[d] NS, Not significant.

mized. The adoptively immunized TX mice irradiated 8 weeks before challenge expressed a moderate and statistically significant level of protection that was lower ($P < 0.01$) than that seen in comparable mice irradiated at week 0.

Close scrutiny of these data suggests that the expression of adoptive immunity in TX mice irradiated 8 weeks before challenge depended entirely on the fact that strain R1Rv bacilli replicated more freely in the no-cell TX controls, to an extent similar to that seen in mice irradiated at week 0 (Table 2). Analysis of the data disclosed significantly low counts ($P < 0.01$) only in those mice irradiated at week 0.

Antituberculosis adoptive immunity in rats. Adoptive immunity to tuberculosis is well expressed in rats without the use of immunomodulating agents. It was therefore of interest to determine whether rats possess a suppressor mechanism analogous to that observed in mice. The magnitude of the adoptive immune response was examined in two experiments in which thoracic duct lymphocytes from BCG-immunized rats were transferred to syngeneic recipients. In the first experiment, normal recipients were compared with recipients that had been exposed to 500 rads. In the second experiment, normal and TXB recipients were compared.

TABLE 3. Expression of adoptive antituberculosis immunity in normal, sublethally irradiated, and TXB rats

Treatment	Log_{10} viable bacilli per spleen in rats receiving:		Immunity[b]	P
	No cells	2×10^8 cells[a]		
None	5.0453	4.4176	0.6277	<0.01
Irradiation	5.4405	4.0289	1.4116	<0.01
None	5.5653	4.8434	0.7219	<0.01
TXB	5.9739	4.5589	1.4150	<0.01

[a] Obtained from BCG-immunized donors.
[b] See Table 1, footnote *b*.

Normal rats expressed moderate but highly significant levels of adoptive immunity, which were greatly increased in recipients that had received irradiation or TXB treatment (Table 3). As in mice, enhanced protective immunity was attributable to the increased multiplication of strain R1Rv cells in control irradiated and TXB animals and also to better expression of the sensitized cells, in that lower absolute counts of strain R1Rv cells were seen in these recipients.

Effect of normal spleen cells on expression of adoptive antituberculosis immunity. Sublethal irradiation depleted the mice of normal lymphoid cells. If these cells play a role in the suppression of adoptive immunity by whatever mechanism, then their replacement might be expected to quench the effectiveness of the sensitized lymphocytes in a dose-related fashion. This idea was tested in the next experiment.

Groups of recipient mice were exposed to 500 rads, challenged with strain R1Rv, and then given graded doses of sensitized spleen cells, ranging from 3.1×10^6 to 25×10^6 per recipient. The dilutions of sensitized spleen cells were prepared either in PBS or in suspensions of normal spleen cells, as indicated (Fig. 3). It was thought that recipients of sensitized cells admixed with normal cells might express substantially less immunity than the recipients of sensitized cells diluted in PBS, particularly at high cell dilutions. In actuality, the addition of even a large number of normal spleen cells to a small number of sensitized cells had no detectable effect on immunity. Large numbers of normal

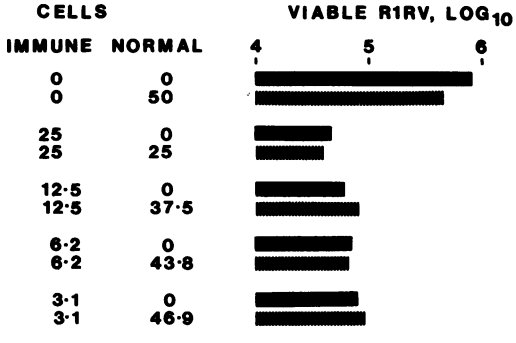

FIG. 3. Adoptive immunity conferred by graded doses of spleen cells from immunized mice. The cells were diluted with either PBS (solid bars) or increasing numbers of normal spleen cells (broken bars). The ratio of immune to normal cells ($\times 10^6$) is shown.

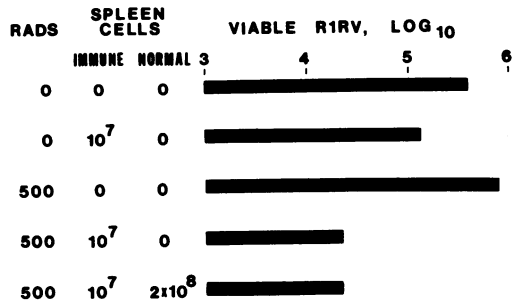

FIG. 4. Failure of 2×10^8 normal spleen cells to affect the adoptive immunity conferred by 10^7 sensitized spleen cells.

spleen cells (50×10^6 or more) inhibited the growth of strain R1Rv cells in irradiated mice to a small but statistically insignificant extent (Fig. 3). Substantial dilution (8-fold) of sensitized lymphocytes produced a disproportionately small decrease (approximately 1.7-fold) in the level of adoptive immunity (20). The sensitized lymphocytes were very potent, since highly significant ($P < 0.01$) levels of adoptive immunity were conferred by as few as 3.1×10^6 spleen cells (Fig. 3).

Reconstitution of irradiated mice. The preceding experiments provided no support for the hypotheses advanced by Mitchison (25) and Dresser (8). A logical alternative was that failure to express adoptive immunity might be due to the action of radiosensitive suppressor cells (22). Attempts were therefore made to reconstitute irradiated mice with possible sources of suppressor cells, namely, the spleen and thymus. The previous experiment had shown that the addition of normal spleen cells to sensitized lymphocytes did not hinder the expression of adoptive immunity. However, the maximum number of normal spleen cells employed, approximately 5×10^7, was lower than the number present in a normal mouse spleen, approximately 7.5×10^7. The effect of transferring a large number of normal spleen cells was therefore examined. The number chosen, 2×10^8 cells, was nearly equivalent to the number present in three normal mouse spleens, but these normal cells had no significant effect on the level of adoptive immunity conferred by 10^7 sensitized spleen cells (Fig. 4). In this particular experiment, the sensitized spleen cells conferred a low (0.46 $\log_{10}$ CF) but significant ($P < 0.01$) level of protection on unirradiated recipients. In an analogous experiment, 10^7 sensitized spleen cells were injected into irradiated recipients with or without 2×10^8 thymus lymphocytes obtained from 5-week-old normal mice. Once again, the normal cells had no effect on the expression of adoptive immunity by sensitized cells.

CY treatment of recipients. CY is an immunomodulating agent that acts in two ways. At a high dose (200 mg/kg), it has a toxic effect on bone marrow precursor cells and peripheral lymphoid cells; at a low dose (20 mg/kg), the bone marrow is unaffected but a suppressor T cell population is selectively eliminated (3). It appeared to be possible that the latter cell population might mediate the suppression of adoptive immunity. Accordingly, groups of recipient mice were treated either 1 or 2 days before cell transfer with either the high or low dose of CY i.v.; another group of recipients was irradiated immediately before cell transfer; and a final group was given neither irradiation nor CY. After challenge with strain R1Rv, half the mice were given 10^7 sensitized spleen cells.

The results obtained with CY treatment were similar regardless of whether the drug was administered 1 or 2 days before cell transfer. As in other experiments, sublethal irradiation enhanced the growth of strain R1Rv cells in control mice ($P < 0.01$), an effect not observed after treatment with either dose of CY (Table 4). The numbers of strain R1Rv cells recovered from adoptively immunized mice that had been irradiated or treated with 200 mg of CY per kg were virtually identical. The magnitude of adoptive immunity in irradiated mice appeared to be greater simply because strain R1Rv grew more freely in the corresponding controls (Table 4). Mice treated with 20 mg of CY per kg responded like untreated recipients, indicating that the suppressor mechanism was not sensitive to low doses of CY.

DISCUSSION

Despite the widespread use of sublethal irradiation to facilitate the expression of adoptive immunity in recipients, little has been published on the mechanism underlying this phenomenon. The early investigators (8, 25) believed that the

TABLE 4. Effect of pretreating recipients with CY on the expression of antituberculosis adoptive immunity

Treatment	$\log_{10}$ viable bacilli per spleen in mice receiving:		Immunity[b]	P
	No cells	10^7 cells[a]		
None	5.3508	5.1377	0.2131	NS[c]
Radiation (500 rads)	6.0884	3.5840	2.5044	<0.01
CY (200 mg/kg)	5.3851	3.5782	1.8069	<0.01
CY (20 mg/kg)	5.0692	4.8608	0.2084	NS

[a] Obtained from BCG-immunized donors.
[b] See Table 1, footnote *b*.
[c] NS, Not significant.

donor cells were competing with the recipient lymphoid cells for space, antigen, or both. Consequently, expression of the donor cells was favored by reduction of the recipient lymphoid cell pool. Evidence for this idea was supported by the observation that supplementing immune donor cells with normal donor cells diminished the adoptive immune response in sublethally irradiated recipients (8). More recently, the generation of cytotoxic lymphocytes in actively immunized mice has been augmented by sublethal irradiation, and this immunopotentiation was suppressed by the injection of normal spleen, thymus, lymph node, or bone marrow cells (26). Similarly, active cell-mediated immunity to *Leishmania tropica* was facilitated by sublethal irradiation and somewhat suppressed by normal spleen cells (14). These results were explained by invoking the elimination of a putative radiosensitive suppressor cell.

In the studies reported here, the earlier hypotheses (8, 25) were tested in experiments in which irradiated recipients were reconstituted with very large numbers (at least two donor equivalents) of spleen or thymus cells. If physical competition between sensitized and nonsensitized lymphocytes were a significant factor, then surely the normal cells would have crowded out the sensitized cells, but this was not the case. By a process of elimination, the alternative hypothesis that a suppressor cell population blocks the expression of adoptive immunity (22) is thereby strengthened.

There are two identifiable components to the enhanced adoptive immunity observed in immunosuppressed recipient mice. First, there was greater multiplication of strain R1Rv cells in the spleens of irradiated control (no-cell) mice than in comparable unirradiated mice. This component, which may be attributable to T cells that participate in the primary immune response to strain R1Rv, was eliminated by irradiation and TXB treatment, but not by CY, cortisone, or TX treatment (Fig. 1 and Table 4). The injection of normal spleen cells appeared to neutralize this effect (Fig. 3). The second component was the elimination of a T lymphocyte population that actively suppressed the expression of adoptive immunity without affecting the multiplication of strain R1Rv cells. All the immunosuppressive maneuvers, including cortisone, high-dose CY, and TX treatments, affected this second population of T lymphocytes, the true suppressors.

The most troublesome aspect of this study was the failure of normal lymphoid cell populations to reconstitute the suppressed state that exists in normal unirradiated recipients. Part of the problem may lie in the nature of the adverse effects of 500 rads of total-body irradiation. Such treatment damages both T and B lymphocytes at various stages of development in the bone marrow, thymus, and peripheral lymphoid organs (1). Moreover, irradiation also affects the bone marrow precursors of monocytes and macrophages that are indispensable to the expression of antituberculosis immunity (1). Consequently, the failure of normal spleen and thymus cells to reconstitute such animals may be due to a lack of resident lymphoid cells, with which the donor cells need to interact to express suppressor activity. This hypothesis is consistent with the complex cellular interactions which regulate other immune responses, in which T lymphocyte subsets are heavily involved (6).

The failure to reconstitute suppression by cell transfer has greatly hindered identification of the cell populations that mediate suppression. It appears that the responsible cells are T lymphocytes, because suppression is not expressed in TXB mice, in whom B lymphocyte and macrophage functions are essentially normal. Further evidence that macrophages are not involved was derived from the observation that sublethal irradiation of TXB recipients did not further enhance the expression of adoptive immunity. The suppressor cells were also resistant to low doses of CY, but sensitive to high doses of CY, to cortisone, and to 500 rads of ionizing radiation.

Other properties of the suppressor cells can be inferred from the experiments in which TX mice were used as recipients. Suppression of adoptive immunity decayed slowly (24), indicating that the mediator cells are long-lived and probably belong to the recirculating pool (11). The suppressor cells regenerated spontaneously after 500 rads of radiation, indicating that precursor cells are resistant to that dose (10). Those precursors are sensitive to 900 rads of radiation, otherwise suppression would have been expressed in the TXB mice. It follows that radiation-resistant T cells (15) cannot regenerate these suppressors, at least not when the thymus is absent.

The negative results of the reconstitution experiments seem to erect an insurmountable barrier to further analysis of the system. One way around this impasse is to attempt to reconstitute suppression in TXB recipients, in whom the problem of bone marrow depletion no longer exists. Even so, optimism should be guarded because in other models normal spleen cells have been shown not to reconstitute suppression in such hosts (4, 9). However, neonatal thymocytes (23) may be effective. This line of investigation is currently being pursued.

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