THE DIFFERING SURVIVAL OF NORMAL AND SENSITIZED SPLEEN CELLS TRANSFERRED TO ALLOGENEIC HOSTS

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The reaction between immunologically reactive cells and a sensitizing antigen has been studied in several different contexts. The direct action of antigen with cells actually or potentially capable of producing antibody may under some circumstances lead to growth stimulation (1) or under different conditions to death of the cells and curtailment of antibody production (2). Both macrophages (3) and lymphoid cells (4) have been found to be damaged by exposure to the sensitizing antigen. Gorer and Boyse have suggested that spleen cells from C57BL mice immunized against A strain antigens may be destroyed by "allergic death" following injection into their respective F1 hybrids (2). Recently it was found that the capacity of C57BL spleen cells to induce runt disease in newborn Swiss mice was reduced if the spleen donors were previously immunized with antigens from the prospective recipients. This was attributed to the deleterious effect on the allogeneic spleen cells resulting from exposure to the antigens of the host and from the immune response of the host (5).

To add to interpretive difficulties surrounding allergic death, about which relatively little is known, lymphoid or spleen cells from different strains of mice have been found to react differently, especially when injected into a hybrid host (2). In an attenpt to gain more information about the disposition of transferred lymphoid cells, we have compared the fates of normal and specifically sensitized C57BL and C_3H spleen cells in normal and irradiated allogeneic hosts and analyzed the respective roles of host and donor cells in the accelerated destruction of sensitized cells.

Materials and Methods

Experimental Animals and Tumors.—Adult male C_3H (H-2k) and C57BL (H-2b) mice taken from the Duke Inbred Colony were used as donors and recipients of spleen cells. The

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C_3H/St mice were obtained by Dr. D. B. Amos from Dr. T. S. Hauschka and originated with Dr. L. C. Strong. The C57BL mice were obtained by Dr. D. B. Amos directly from the Springville Colony in 1958. The transplantable tumors used for immunization were the ascites carcinoma BP8 indigenous to the C_3H strain, the ascites lymphoma EL24 indigenous to the C57BL strain, and QL1, a lymphoma from the DBA/1 (H-2q) strain.

Spleen Cell Suspensions.—Spleen cell suspensions were prepared by removing the spleen tips, gently teasing the cells into 1 ml of Ringer's solution, and passing the suspension through a graded series of No. 22 to No. 30 guage needles. After each suspension was allowed to stand for about 10 minutes to dispose of particles and clumps by settling, the upper portion was removed for use. The cells were counted and the percentage staining with 1:2000 trypan blue determined. The proportion of damaged cells varied between 10 and 15 per cent. Suspensions were injected into tail veins in volumes of 0.5 to 0.7 ml per mouse, with an average of 1×10^8 spleen cells injected per mouse. At various time intervals after injection the recipients were sacrificed by cervical dislocation, their spleens excised and weighed, and a cell suspension prepared as described above. The total number of cells per spleen was counted and cell origin determined by differential cytotoxicity and radioautography techniques.

Immunization.—Reciprocal isoimmunization was accomplished by immunizing with a single intravenous injection of 2 to 5 million ascites tumor cells. C_3H mice were immunized against the C57BL tumor and C57BL mice against the C_3H tumor. Five to 7 days later the mice were killed to provide spleen cell suspensions.

Immunization against an indifferent antigen was performed in a control group of C57BL donors which were injected intravenously with either 2×10^8 washed sheep red blood cells or 5×10^6 QL1 cells. Mice were killed 5 to 7 days later to provide spleen cell suspensions.

Antisera.—Isoantisera against C_3H antigens were prepared in C57BL mice by repeated inoculations of the BP8 tumor, while isoantisera against C57BL antigens were prepared in C_3H mice by inoculations of the EL24 tumor. The schedule for both strains consisted of (*a*) an initial inoculation of 1 to 2×10^6 ascites cells injected subcutaneously in the right thigh region, (*b*) subsequent doses of 5×10^6 cells injected intraperitoneally at 3- to 4-week intervals, and (*c*) withdrawal of blood samples from the retroorbital venous plexus on the 5th or 6th day following one of these booster injections. Blood from six animals was pooled and the sera thus obtained divided into 0.025 ml amounts and stored at −20°C.

Serological Typing of the Spleen Cells.—Boyse's modification of the cytotoxicity test of Gorer and O'Gorman (6) was used. A 0.025 ml aliquot of a suspension containing 1 to 2×10^6 spleen cells per ml was added to 0.025 ml of a 1:20 dilution of antiserum. After 10 minutes of incubation at room temperature, 0.025 ml of guinea pig complement was added. Following the 1 hour incubation at 37°C, 0.05 ml of supernatant was pipetted from each tube, 0.025 ml of a 1:2000 solution of trypan blue added, and the number of stained and deformed cells determined (non-viable cells). In each individual test the percentage of viable and non-viable cells was determined in six different media,—in each of the two test isoantisera alone or with added complement, in balanced salt solution, and in complement alone. The results obtained in the four control media in each experiment were always quite similar.

The results of the cytotoxicity tests were expressed not only as percentages of stained or damaged cells but also as cytotoxic indices according to the formula (7):

$$\text{cytotoxic index} = \frac{\text{per cent non-viable (experimental)} - \text{per cent non-viable (control)}}{\text{per cent viable (control)}} \times 100$$

The control values were those of the complement control.

Irradiation.—Whole body irradiation was delivered at the rate of 116 roentgens per minute for a total of 900 r given as a vertical beam from a Westinghouse machine under the following conditions: 280 kvp, 20 ma, 50 cm distance, ½ Cu + Al HVL (half value layer), 1.44 Cu

(open field). During radiation the mice were contained in a rectangular perforated polyethylene box. Cell suspensions were injected 3 hours later.

Radioautography.—Mice received 3.5 μc tritiated thymidine, specific activity 1.9 c/mmole/gm body weight. The total dose was divided into four intraperitoneal injections over a 2½ day period, with the last injection administered 3 to 5 hours prior to death. Mice subjected to immunization received the same course of four injections of tritiated thymidine beginning 3 days after antigen administration and 2½ days before sacrifice.

Brush preparations of spleen were prepared as follows: fine camel hair brushes were dipped into normal mouse serum and brushed gently over areas of freshly cut spleen. Smears were then made on clean glass slides and immediately fixed in absolute methanol. Bone marrow was prepared in a similar manner. Sections of spleen, liver, lung, small bowel, thymus, skin, lymph nodes, and pancreas were taken and fixed in phosphate-buffered formalin. Five-micron paraffin sections of these tissues were rehydrated, as were the spleen and bone marrow smears, prior to dipping in Kodak's NTB-2 radioautographic emulsion equilibrated for 1 hour at 43°C. Slides were stored in a CO_2 light-tight chamber for 3 weeks, at which time they were developed and stained with hematoxylin. The proportion of labeled cells were determined for each donor and each recipient. A minimum of 3 to 5 grains per nucleus, depending on the background, was considered as significant. No less than 10,000 cells were counted per slide.

Cell Mixture Experiments.—Cell suspensions from spleens of normal and previously sensitized C57BL mice were prepared as previously described. Varying proportions of these cells, ranging from 5 per cent sensitized and 95 per cent normal to 50 per cent sensitized and 50 per cent normal were mixed, held at room temperature for 30 minutes, and then injected into the tail veins of C_3H mice. An average of 1×10^8 spleen cells were transferred per animal. After 2 days, recipients were killed and their spleens removed and analyzed by a differential cytotoxicity test.

A similar procedure was followed in mice injected with tritiated thymidine in which mixtures of labeled, normal C57BL spleen cells and non-labeled, sensitized C57BL spleen cells were prepared and injected. Two days after intravenous transfer of a mixture of 1×10^8 cells to C_3H mice, the animals were killed. In addition to serological typing, the origin of the spleen cells was analyzed radioautographically.

Normal Spleen Cells Plus Antisera.—Six adult male C_3H mice received 1×10^8 normal C57 spleen cells intravenously. The donor cells were incubated for 30 minutes at room temperature with 0.5 ml of a 1:20 dilution of the C57BL anti-C_3H antiserum prior to injection. Recipient animals were sacrificed 2 days later and their spleens typed.

Statistical Methods.—The t test was used in an analysis of the significance of the difference in cytotoxic titers between the various experimental groups.

RESULTS

Survival of Transplanted Cells Assessed By Differential Cytotoxicity.—

Fate of normal spleen cells in allogeneic hosts: Spleen cells suspensions were tested with each of the two test isoantisera and the total of damaged cells equaled 96 to 100 per cent. In both combinations, (C57BL → C_3H; C_3H → C57BL) similar results were obtained. The percentage of C57BL cells in C_3H hosts expressed as a mean of the cytotoxic index from 0 to 24 hours was 17 per cent; from 24 to 48 hours the mean was 15.3 per cent. Corresponding values for C_3H spleen cells in C57BL hosts were 15.1 and 16.3 per cent. (The actual values after subtraction of the stained cells in controls are depicted in Figs. 1 and 2.)

Thereafter, values declined rapidly so that after 3 to 4 days donor type cells were no longer detectable.

Fate of sensitized cells in allogeneic hosts: Presensitization of C57BL or C_3H donor spleen cells against the tissues of the other strain led to their accelerated disappearance from the spleen when transferred into allogeneic hosts. Figs. 1 and 2 show that although initially these cells were present in approximately the same proportion as cells from normal animals (mean cytotoxic index in both combinations for the first 24 hours was 14.3 per cent) they disappeared very rapidly and after 1 day were no longer detectable. The difference between

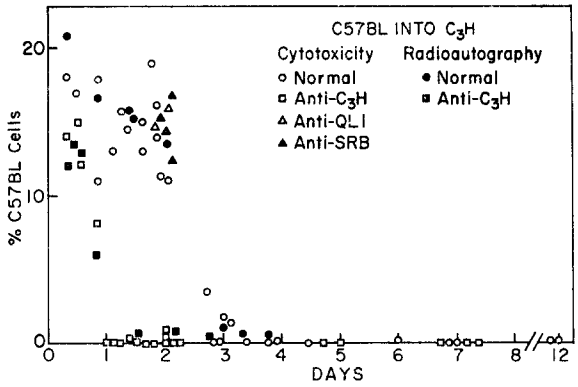

FIG. 1. Per cent survival after subtraction of control values of normal and presensitized C57BL spleen cells when transferred into C_3H hosts as determined by differential cytotoxicity and radioautography at varying time intervals. (For details of presensitization and experimental procedures see text.)

normal and immune donor cells in the 1- to 2-day interval was highly significant ($P < 0.001$).

Spleen weights of recipients of both normal and immune cells rose rapidly during the first 3 days to over twice control values. It is noteworthy that while the spleen weight of the recipients of normal cells returned to normal within a week, the spleens of recipients of immune cells maintained and even slightly increased their weight over this period of time.

The accelerated disappearance of immune cells could be due to their allergic death following contact with the antigens of the host. However, since presensitized cells constituted a stronger stimulus for the increase in spleen weight, it was possible that the sensitized allogeneic cells stimulated a more vigorous and rapid immune response of the host. To test these possibilities two experiments were carried out. In one, the donor was immunized against an indifferent antigen,—QL1 or sheep red blood cells. In the second, the donor cells were injected into lethally irradiated hosts. The recipients were killed after 2 days,

since the clearest difference between immune and normal donor spleen cells was evident at this time. The survival of spleen cells immunized against an indifferent antigen in allogeneic hosts (Fig. 1) was similar to that of normal spleen cells ($P > 0.5$) but definitely greater than spleen cells immunized against their host ($P < 0.001$). As demonstrated in Fig. 3, lethal irradiation of the host completely eliminated the differences in the disappearance rates of normal and immune cells ($P > 0.5$).

Radioautographic findings: As shown in Figs. 1 and 2, results obtained after radioautographic analysis of donor recipient preparations confirmed the cyto-

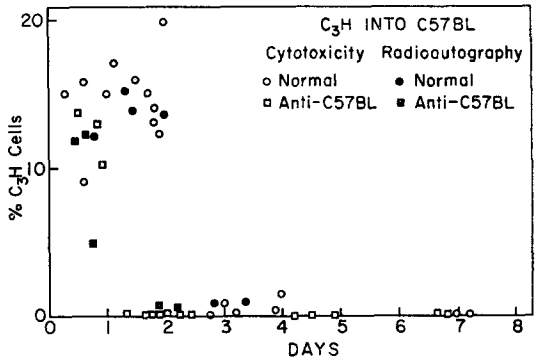

FIG. 2. Per cent survival after subtraction of control values of normal and presensitized C_3H spleen cells when transferred into C57BL hosts as determined by differential cytotoxicity and radioautography at varying time intervals. (For details of presensitization and experimental procedures see text.)

toxic data. A correction factor depending on the proportion of labeled cells present in the donor spleen suspensions was applied to the proportion of labeled cells present in the recipient spleens. For example, 25 per cent of the cells in a C57BL donor spleen were found to be labeled at the time of injection and 3.8 per cent of recipient spleen cells were labeled 24 hours after injection. Thus, the actual proportion of C57BL cells in this instance was computed as $\frac{3.8}{25} \times 100$ or 15.2 per cent.

Experiments in Which Normal and Sensitized Cells Were Mixed in Varying Proportions.—Having found a system in which we could distinguish between a sensitized and a normal cell population, the possibility of investigating the effect that relatively few cells can have upon a large population of other cells arose. Consequently, mixtures ranging from 5 per cent sensitized and 95 per cent normal to 50 per cent sensitized and 50 per cent normal were prepared and transferred into C_3H mice. When spleens from C_3H recipients were analyzed 2 days later by differential cytotoxicity and radioautography, the results

indicated that as little as 5 to 10 per cent of the donor cell population need be sensitized for the mixture to behave as if it were obtained from a sensitized animal (Fig. 4).

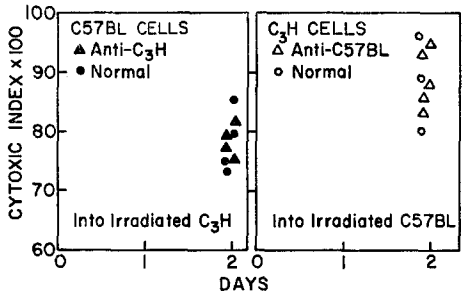

FIG. 3. Survival of C56BL spleen cells in lethally irradiated C_3H hosts and of C_3H spleen cells in lethally C57BL hosts at the 2-day interval. The results are expressed as cytotoxic index according to the formula:

$$\text{cytotoxic index} = \frac{\text{per cent non-viable (experimental)} - \text{per cent non-viable (control)}}{\text{per cent viable control}}$$

(For methods of x-irradiation and experimental procedures see text.)

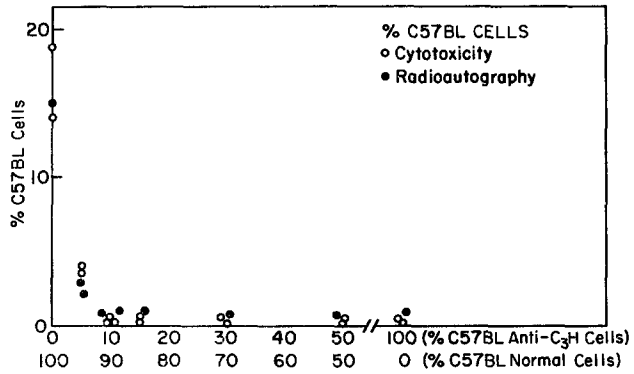

FIG. 4. Per cent survival after subtraction of control values of mixtures of normal and presensitized C57BL spleen cells when transferred into C_3H mice and analyzed at the 2-day interval. (For details of experimental procedures see text.)

Normal Spleen Plus Antisera.—The possibility that spleen cells from sensitized animals are damaged by a reaction involving extrinsic antibody with extrinsic antigen at the cell surface has been suggested (3). To further explore this possibility, normal C57BL spleen cells were incubated for 30 minutes with C57BL anti-C_3H antibody and transferred into C_3H mice. Recipients were sacrificed 2 days later and cytotoxic analysis of the spleens carried out. No

difference was found between the animals which received normal C57BL spleen cells and those which received normal spleen preincubated with C57BL anti-C_3H antisera ($P > 0.5$).

DISCUSSION

Consistent with previous reports, the survival time of homografts of lymphoid cells in allogeneic hosts proved to be short (8). In the present study, spleen cells from normal mice injected intravenously into adult allogeneic hosts could be demonstrated in the recipient spleen for 3 days. Thereafter they were no longer detectable. In contrast spleen cells from mice sensitized against antigens of the prospective host disappeared far more rapidly and could not be detected in the recipient's spleen later than 1 day after injection. This was established both by serological typing of the host's spleen cells and by isotope labeling of donor cells later identified in the recipient's spleen.

Concomitant with rapid disappearance of the sensitized spleen cells was a marked increase in spleen weight. The splenomegaly after injection of immune cells was considerably greater than that found after injecting normal cells. Splenic hypertrophy following injection of foreign cells without proliferation of foreign cells has been found in runt disease (9) and in F1 homologous disease (10).

Radioautographic observations revealed that the fate of allogeneic cells in the liver, unlike those in the spleen, was unaffected by prior sensitization. In both cases, labeled allogeneic cells formed periportal cell infiltrates, and in both cases the label was of equal intensity at 2 days. Miller *et al.*, have (11) furnished evidence that these infiltrates constitute a reaction of the injected cells against the host. Similar infiltrates have been described by Gorer and Boyse, following injection of parental cells into F1 recipients (2). The differences in the fate of injected lymphocytes in the liver and those in spleen have been observed by Osogoe (12) who found periportal accumulations of lymphocytes in the liver of rabbits persisting long after they had disappeared from the spleen. Fichtelius (13), whose results complement those of Osogoe, reported that 24 hours after transfusion of P^{32}-labeled lymphocytes, activity from injected lymphocytes could be found in the spleen and liver after syngenesiotransfusion (rat-to-rat, the same litter) but only in the liver after heterotransfusion (rabbit-to-rat).

In our experiments, the accelerated disappearance of sensitized cells from the spleen was considered due to their increased rate of destruction. Dilution of label was not considered significant at the 1 to 2 day interval. A careful search for labeled cells in other organs yielded no information suggesting the recirculation and lodging in other tissues of donor lymphocytes. As mentioned previously, the liver did contain periportal accumulations of labeled cells. However, no increase in this label was noted to account for its disappearance from the spleen.

It has been demonstrated that peritoneal macrophages from guinea pigs in

which a delayed hypersensitivity reaction to various antigens has been induced are damaged by contact with antigen (3). McKhann (14) has shown that in mice previously immunized against allogeneic transplantable tumors the intraperitoneal graft of the tumors results in an increased destruction of peritoneal lymphoid cells. Gorer and Boyse (2) found that spleen cells from mice of one strain immunized against antigens of mice of another strain were rapidly destroyed following injection into the corresponding F1 hybrids; they suggested "allergic death" of the cells following contact with the antigen as a possible mechanism. Similarly, accelerated disappearance of the sensitized spleen cells in our experiment could have been due to allergic death of the injected cells following contact with antigens of the host. The fact that immunization of donors with sheep red blood cells or with antigens of the third unrelated strain of mice did not cause an accelerated destruction of the cells indicated that specific sensitization against host antigens was a prerequisite for their rapid elimination. On the other hand, the prevention of accelerated destruction by prior lethal whole body irradiation of the recipients and the persistence of immunized cells in the host point to an important role played by the host in the rapid destruction of the sensitized cells. While the results of the irradiation experiments could be explained by reduced antigenicity of the host following irradiation, this seems unlikely since irradiation of mouse tissues *in vitro* has no appreciable effect on their antigenicity (15).

The finding that the spleens of recipients of sensitized cells were larger than those receiving normal cells suggests that sensitized cells may constitute a greater antigenic stimulus than non-sensitized cells. It is interesting in this respect that Basch and Stetson found that stimulation of the murine reticuloendothelial system in response to intravenous injections of BCG increased the specific isoantigenic acitvity of spleen (16). Similarly, preliminary studies in our laboratory indicate that spleen cells from a previously immunized host are more antigenic than cells from an unsensitized animal regarding both their capacity to absorb antibody and their ability to elicit early antibody formation (17).

Thus, our experiments showed conclusively that the response of the host eliminates specifically sensitized cells more rapidly than non-sensitized cells. One cannot exclude, the possibility however, that in normal recipients damage to sensitized cells by contact with antigens of the host may play a role in their rapid destruction.

It is possible that the response of the host may be accelerated by cell breakdown products liberated as a result of a reaction of the sensitized cells against the host. Such a mechanism of acceleration of immune response by cell destruction has been postulated by Braun (18).

Since it is known from the studies of Jerne (19) that only a small number of lymphoid cells from a specifically immunized host participate in an immune

reaction, one wonders how sensitization of a limited number of cells would result in the destruction of the whole population when transferred to the recipient to whose antigens sensitivity had been produced. David and Lawrence have investigated delayed hypersensitivity in an *in vitro* system designed to elucidate the effects of antigen on migration of peritoneal exudate cells obtained from guinea pigs with delayed allergy (20). In studies on the inhibition of cell migration by antigen, they found that mixing as few as 2.5 per cent cells from an immune animal with 97.5 per cent normal cells causes the whole population to react as if it were immune. Similar results were obtained in our experiments in which sensitized and normal cells were mixed. As few as 5 to 10 per cent sensitized cells were needed to cause an accelerated disappearance of the entire cell mixture from the allogeneic spleen. While it is tempting to draw analogies, the two experimental situations were quite different. This extremely interesting phenomenon can perhaps be shown in a variety of systems, however, possibly explaining the failure of many investigators to demonstrate cells transferred from an immune donor at the site of a hypersensitivity or skin graft reaction in a challenged normal host (21, 22).

The mechanism whereby relatively few sensitized cells can affect a large population of normal cells remains unknown. Although this phenomenon has not been clarified in the experiments reported here, two factors should be considered: (*a*) either only 5 to 10 per cent sensitized cells are needed in the mixture for the host to respond in an accelerated fashion and reject the entire population or (*b*) a cell-to-cell transfer of specific information may take place either at long range through some humoral substance or following actual cellular contact between immune and normal cells (23).

Experiments to better delineate the first possibility are in progress. Whether the host would respond to only the immune cells in a vigorous and rapid fashion, with death of normal cells occurring secondary to release of cellular enzymes (23), or whether, once stimulated by sensitized cells, the recipients response is accelerated toward the entire foreign cell mixture is under current investigation.

SUMMARY

Normal and isoimmune C_3H and C57BL spleen cells were transferred intravenously into normal and irradiated allogeneic recipients and the fate of the donor cells determined by differential cytotoxicity and radioautographic techniques. It was found that spleen cells sensitized against their prospective recipients could be identified in the host's spleen for 1 day, whereas normal donor cells survived 2 to 3 days. Spleen cells presensitized with an indifferent antigen had survival times similar to normal donor cells. Irradiation of the recipients prior to cell transfer eliminated any difference in survival times between normal and presensitized donor cells in allogeneic hosts. It is concluded

that the host plays an important role in the rapid destruction of spleen cells presensitized against host antigens.

Experiments in which sensitized and normal C57BL spleen cells were mixed and transferred into normal C_3H mice indicated that as little as 5 to 10 per cent of the donor cell population need be sensitized for the entire mixture to behave as if it were obtained from a sensitized animal, as shown by its elimination in the 1-day interval.

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