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Difference in the Target Cells for Tolerance Induction in Relation to the Dose of Tolerogen

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Summary. Bone marrow cells and thymus cells were observed in cell transfer experiments to collaborate in the production of anti-BSA antibodies. The target cells for tolerance induction either with a 'low dose' of antigen (100 μg of deaggregated BSA once a week ×5) or with a 'high dose' (a single injection of 5000 μg) were identified by the same technique. In 'low dose' tolerance, some indication was obtained that thymus-derived cells in peripheral lymphoid systems were the target cells; bone marrow-derived cells appeared not to be so susceptible, and the cells residing in thymus or bone marrow seemed to remain unimpaired. In contrast, the injection of a 'high dose' of tolerogen rendered both types of cells in spleen, thymus cells and bone marrow cells, unresponsive or hyporesponsive in parallel with one another.

INTRODUCTION

Collaboration between thymus-derived cells (T cells) and bone marrow or non-thymus-derived cells (B cells) has been shown to be one of the important initial steps in the antibody response of mice to various antigens (Claman and Chaperon, 1969; Mitchell and Miller, 1968; Taylor, 1969; Chiller, Habicht and Weigle, 1970; Miller and Cudkowicz, 1971). The evidence shows that the antibody producers are B cells, although T cells also intereact specifically with antigens (Mitchell and Miller, 1968; Nossal, Cunningham, Mitchell and Miller, 1968; Claman and Chaperon, 1969).

Different experimental results have been presented to suggest that the target cells for tolerance induction may be thymus cells (Isaković, Smith and Waksman, 1965; Taylor, 1969), bone marrow cells (Playfair, 1969), both thymus and bone marrow cells (Chiller *et al.*, 1970; Chiller, Habicht and Weigle, 1971), or T cells in the pool of recirculating lymphocytes. Conceivably, such a discrepancy can be ascribed to the difference in experimental conditions; for instance, kinds or doses of tolerogenic antigen, or the use of immunosuppressive drugs. Recently, Mitchison (1971) presented experimental results which suggest that both T cells and B cells are made unresponsive in his 'high zone' tolerance, but only T cells were the target in his 'low zone' tolerance.

In the preceding paper, the conditions for inducing tolerance by deaggregated BSA have been established (Katsura, 1972b), and it was shown that multiple weekly injections of 100 μg abrogated the anti-BSA response of mice, and that a single injection of 5000 μg was not so effective with a slight priming of γG-antibody response. The present paper is concerned with the identification of target cells for the induction of such 'low dose' and 'high dose' tolerance. A difference in the target cells according to the dose of tolerogen is demonstrated.

MATERIALS AND METHODS

Mice

Male and female CBA/St Ms mice (originally obtained from National Institute of Genetics, Mishima, Japan), aged 7–8 weeks, weighing about 25 g, were used.

Antigen

Crystalline bovine serum albumin (BSA) (Pentex Inc., U.S.A.) was used as an antigen throughout the experiments. Soluble BSA (sBSA) deaggregated by ultracentrifugation was used as tolerogen, and alum-precipitated BSA (AP-BSA) with or without endotoxin (ET) (Lipopolysaccharide B_4, extracted from *Escherichia coli*, 0111; Difco Lab. U.S.A.) was used for immunization. AP-BSA and sBSA were prepared as in the previous papers (Katsura, 1972a, b).

(a) *Tolerance induction*

Mice were given either five intravenous injections of sBSA at 100 μg once a week or a single intravenous injection of 5000 μg of sBSA. Ten days later, they served as donors of lymphoid cells. Multiple injections of 100 μg and a single injection of 5000 μg are designated in this paper as 'low dose' and 'high dose', respectively.

(b) *Immunization*

In these experiments, the immunization was performed twice to the recipients of lymphoid cells. Primary immunization was performed with 100 μg of AP-BSA intravenously 1 day after the time of cell transfer and a secondary immunization was done 10 days later with 100 μg of AP-BSA and 10 μg of ET.

Irradiation

Mice used as recipients were given 800 R whole body irradiation with a therapeutic ^{60}Co γ-ray source (Type RTGS-2, Shimadzu, Kyoto, Japan) at the rate of 55 R/min. Irradiated mice were given pencillin in the drinking water (200,000 units/l).

Preparation of cell suspensions

Mice were killed in a CO_2-asphyxiator. Eagle's minimum medium containing 60 μg Kanamycin/ml was used for suspending and washing cells. All procedures were done in or on crushed ice. Bone marrow was pushed out from both femurs and tibiae by hydrostatic pressure using a syringe with a 22-gauge needle. The mixture of bone marrow and the medium was ground with a pestle on a stainless steel sieve. Thymuses and spleens were chopped quickly with scissors, ground with a pestle on the sieve, and poured through with medium. Further disruption was achieved by gentle aspiration with a Pasteur pipette followed by filtration through cotton wool. Single cells thus obtained were washed twice by low-speed centrifugation with Eagle's medium, resuspended, and counted with a Thoma's haemacytometer. Viability of nucleated cells was estimated by the trypan blue-exclusion test. Cells in 0·5 ml of Eagle's medium were injected intravenously (except where indicated) into mice receiving 800 R γ-ray irradiation 24 hours previously. When cells from tolerant donors and those of normal donors were used in an experiment, normal cells were injected 24 hours after the injection of tolerant donor cells.

Test for antibody

Mice were repeatedly bled *via* the retro-orbital plexus. Anti-BSA titration was performed by a passive haemagglutination test, according to the procedure described previously (Katsura, 1972a). All titrations were performed with a 'blind' system, the observer not knowing the source of the serum samples. In this paper, 2-mercaptoethanol (2-ME)-sensitive and 2-ME resistant antibodies are designated as γM and γG antibodies, respectively.

RESULTS

NORMAL SPLEEN CELLS

Groups of four to eight irradiated mice each were inoculated with normal spleen cells. Inoculation sizes were 3×10^7, 2×10^7, or 3×10^6 cells per recipient mouse. The recipients were immunized with 100 μg of AP-BSA on the next day and 100 μg of AP-BSA + 10 μg of ET 10 days later. The adequacy of this immunizing schedule (designated as the standard procedure in these experiments) for the adoptive immune response was checked by finding in a preliminary test that it elicited strongly both γM and γG antibodies. As shown in Fig. 1, the level of both γM and γG antibodies increased with the number of

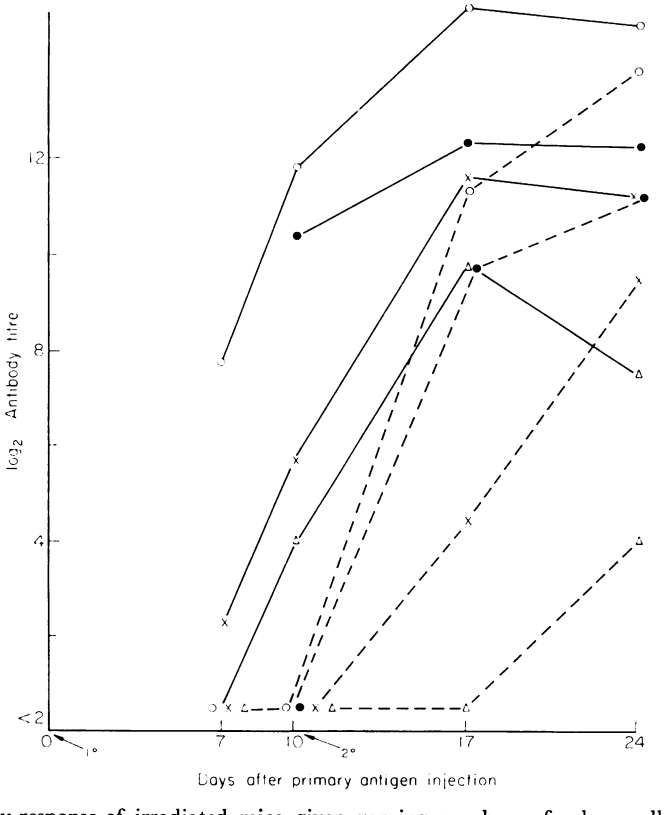

FIG. 1. Antibody response of irradiated mice given varying numbers of spleen cells from normal syngeneic donors. The numbers of spleen cells given per recipient were ○, 3×10^7; ●, 2×10^7; ×, 1×10^7 or △, 3×10^6. Each point refers to the geometrical mean titre of six to ten recipient mice. Solid and broken lines represent total and 2-ME-resistant antibodies, respectively. Primary immunization (1°) was performed 1 day after the cell transfer and secondary immunization (2°) 10 days thereafter.

cells inoculated. The antibody produced in response to the first immunization was γM alone, and γG antibodies appeared after the second immunization.

NORMAL THYMUS CELLS AND NORMAL BONE MARROW CELLS

Groups of four to eight irradiated mice each were given 2×10^7 bone marrow cells together with varying numbers of thymus cells (Fig. 2a). In another reciprocal experiment, varying numbers of bone marrow cells were injected along with 8×10^7 thymus cells into irradiated recipients (Fig. 2b). In the cases where 8×10^7 thymus cells were given, 4×10^7 cells were injected intraperitoneally. The recipients were immunized by the standard procedure.

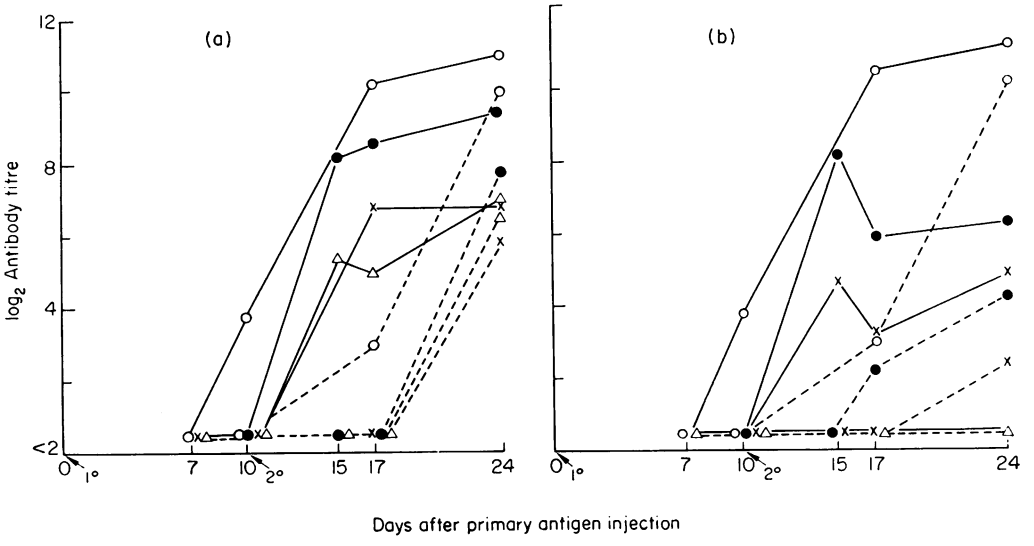

FIG. 2. (a) Antibody response of irradiated mice given a fixed number of bone marrow cells (2×10^7) and varying numbers of thymus cells from normal syngeneic donors. The numbers of thymus cells given per recipient were ○, 8×10^7; ●, 3×10^7; ×, 1×10^7 or △, 0. (b) Antibody response of irradiated mice given a fixed number of thymus cells (8×10^7) and varying numbers of bone marrow cells from normal syngeneic donors. The numbers of bone marrow cells given per recipient were ○, 2×10^7; ●, 5×10^6; ×, 1×10^6 or △, 2×10^5. Each point refers to the geometrical mean titre of five recipient mice. Solid and broken lines represent total and 2-ME-resistant antibodies, respectively. Immunization procedure is the same as shown in Fig. 1.

As shown in Fig. 2a, no antibody was detected in mice given bone marrow cells alone and the addition of thymus cells was required to develop the γM and γG antibody response. With an increase in the number of the added thymus cells, both γM and γG responses were enhanced. The response to the primary immunization was weak, slight γM activity being detected only in the group aided with 2×10^8 thymus cells. Fig. 2b shows the results in a reciprocal experiment in which a fixed number of thymus cells and a varying number of bone marrow cells were inoculated. The response increased with the number of bone marrow cells added. Irradiated mice inoculated only with thymus cells all died within 15 days. These results clearly indicate a synergistic action between thymus and bone marrow cells in the production of both γM and γG antibodies to BSA.

ANAYSIS OF THE TOLERANT STATE INDUCED BY 'LOW DOSE' ANTIGEN

Mice were made tolerant with five-weekly injections of 100 μg sBSA, and used as cell donors 10 days after the last injection. This sBSA treatment has previously been reported to be effective in abrogating the anti-BSA response (Katsura, 1972b). Spleen, thymus, and bone marrow cells were harvested from the normal and tolerant animals and injected into irradiated recipients (six mice in each group) in different combinations. The inoculation sizes were as follows: thymus cells, 8×10^7; bone marrow cells, 2×10^7; normal spleen cells, 2×10^7; tolerant spleen cells, 3×10^7.

As shown in Fig. 3, the spleen cells from tolerant donors were completely incompetent in the anti-BSA response. In this experiment, such incompetent spleen cells were sup-

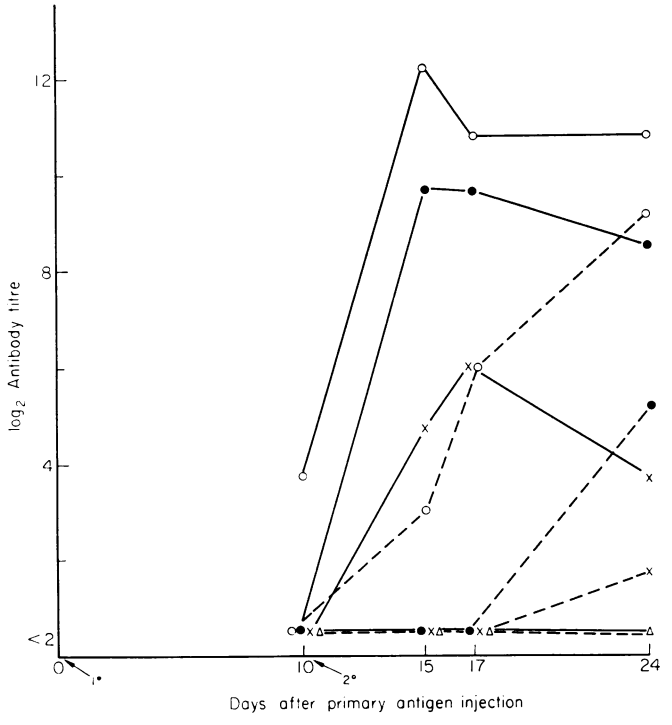

FIG. 3. Antibody response of irradiated mice given 3×10^7 spleen cells from syngeneic donor mice made tolerant by 'low dose' and cells from normal mice. The numbers and source of normal cells are as follows; ○, 2×10^7 spleen cells; ●, 8×10^7 thymus cells; ×, 2×10^7 bone marrow cells or △, no cell. Each point refers to the geometrical mean titre of four to six mice. Solid and broken lines represent total and 2-ME-resistant antibodies respectively. Immunization procedure is the same as shown in Fig. 1.

plemented with cells from normal donors on the following day. The addition of normal spleen cells was most effective. The effectiveness of normal thymus cells came next and normal bone marrow cells conferred only a weak competence.

Fig. 4 shows the interaction between thymus and bone marrow cells taken from tolerant or normal donors. In any combination of thymus and bone marrow cells irrespective of their origin, good responses were obtained. That even thymus and bone marrow cells from tolerant donors were able to collaborate should be noticed.

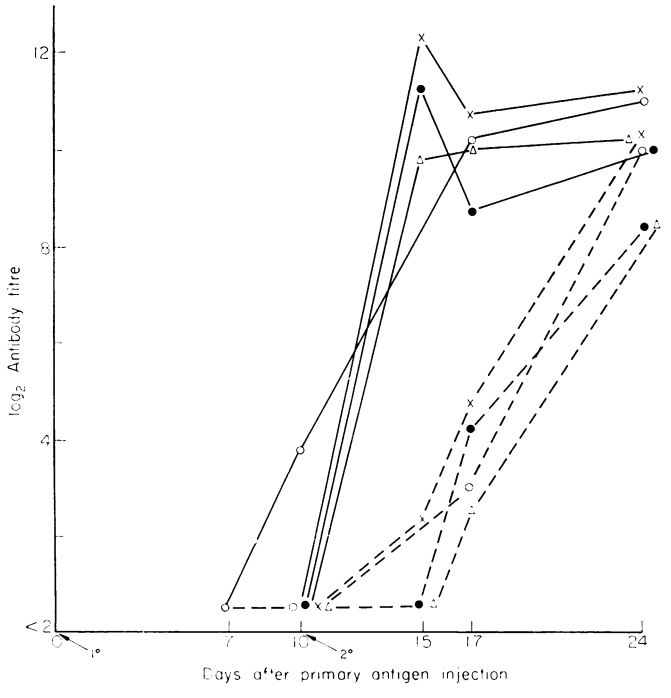

FIG. 4. Antibody response of irradiated mice given thymus and bone marrow cells from mice made tolerant by 'low dose' or from normal mice. The number of thymus cells and that of bone marrow cells are 8×10^7 and 2×10^7, respectively. Each point refers to the geometrical mean titre of six mice. Solid and broken lines represent total and 2-ME-resistant antibodies, respectively. Immunization procedure is the same as shown in Fig. 1. ○, n-T+n-BM; ●, t-T+n-BM; ×, n-T+t-BM; △, t-T+t-BM.

ANALYSIS OF THE TOLERANT STATE INDUCED BY 'HIGH DOSE' ANTIGEN

The experimental design was the same as that used in the cellular analysis of the tolerant state induced by 'low dose' antigen. Cells were harvested from mice given a

TABLE 1

MAXIMUM TITRES OF γM AND γG ANTIBODIES OF INDIVIDUAL RECIPIENT MICE GIVEN LYMPHOID CELLS[a] FROM NORMAL AND 'HIGH DOSE' TOLERANT MICE

Cells inoculated	Max. γM[b]	Max. γG[c]
8×10^7 t[d]-T+2×10^7 n[e]-BM	8,-[f], -,-	-,-,-,-
2×10^7 t-BM+8×10^7 n-T	5,4,-,-	11,3,-,-
3×10^7 t-S+2×10^7 n-BM	8,-,-,-	6,4,3,-
3×10^7 t-S+8×10^7 n-T	8,4,-,-	6,-,-,-
3×10^7 t-S	5,-,-,-	-,-,-,-
8×10^7 lt[g]-T+2×10^7 lt-BM	13,12,12,12,11,10	10,10,9,9,7,6

[a] Thymus cells (T), bone marrow cells (BM) or spleen cells (S).
[b] The higher of the $\log_2$ total antibody titres at day 15 or day 17.
[c] The highest of the $\log_2$ 2-ME-resistant antibody titres within 24 days after the primary immunization.
[d] 'High dose' tolerant donors.
[e] Normal donors.
[f] Negative value.
[g] 'Low dose' tolerant donors.

single injection of 5000 μg sBSA 10 days previously. Spleen, thymus or bone marrow cells of these mice were injected into irradiated recipients in combination with normal cells, and immunized by the standard procedure. The results are shown in Table 1. Since antibodies were undetected in many mice, calculation of mean values seems absurd, so instead the maximum titres of γM and γG antibodies in each mouse were listed. Synergism between thymus cells and bone marrow cells from 'low dose' tolerant mice is also shown.

The results were in marked contrast to the previous results obtained with the mice made tolerant by 'low dose'. Single injection of 5000 μg of sBSA suppressed not only the reactivity of spleen cells but also that of cells in thymus and bone marrow.

DISCUSSION

The restoration of immune capacity of irradiated mice receiving both thymus cells and bone marrow cells from syngeneic donors, first reported by Claman, Chaperon and Triplett (1966), was observed also in this experiment for the anti-BSA response (Fig. 2a and 2b). The collaboration of these two types of cells in the anti-BSA response has already been demonstrated by Taylor (1969). The present results and experimental conditions for cell transfer are essentially the same as those of Taylor. Usually, the minimum number of cells required for cell cooperation seemed larger for thymus cells than for bone marrow cells; bone marrow cells were effective even in doses of the order of 10^5, but thymus cells were needed in the order of 10^7. Further analyses of the synergism of these two types of cells in antibody formation has not been performed, since this experiment was aimed principally at delineating the target cells for tolerance induction, under conditions which had been established previously (Katsura, 1972b).

Different conclusions have been reached about the target cells for tolerance induction, as mentioned in the introduction. This may be attributable to differences in antigen and possibly also to the procedures for tolerance induction, especially in relation to the dose of tolerogen injected. The results in part of the present experiment seem essentially consistent with Mitchison's finding that both B cells and T cells are made tolerant in his 'high zone' tolerance but only the latter is responsible for his 'low zone' tolerance (Mitchison, 1971), though whether 'low dose' and 'high dose' tolerance in the present experiment correspond to his 'low zone' and 'high zone' tolerance (Mitchison, 1964, 1968) is problematical. Spleen cells from mice made profoundly tolerant by 'low dose' (100 μg once a week $\times$ 5) did not confer on the irradiated recipients an ability to produce γM or γG antibodies, in accordance with the adoptive tolerance of Neeper and Seastone (1963) and Argyris (1966). Although the treatment with 'low dose' antigen caused almost complete unresponsiveness of intact mice, it hardly affected the competence of cells residing in either thymus or bone marrow (Fig. 4). The tolerant state thus induced can be ascribed to the diminished activity of T cells in spleen (and probably also in other peripheral lymphoid systems) (see Fig. 3). This agrees to some extent with the results of Miller and Mitchell (1970) who showed that the target cells for tolerance to sheep red blood cells were T cells in the mobile pool of recirculating lymphocytes. It appears that the 'low dose' of tolerogen does not produce a great effect on the B cells in the spleen, since the response was improved only slightly by the addition of normal bone marrow cells to tolerant spleen cells. Another possibility, however, which should also be taken into account is that a large proportion of B cells may be suppressed by the tolerogen, because

even a relatively small number of bone marrow cells was found to cooperate with thymus cells with good efficiency (cf. tolerant spleen cells + normal thymus cells in Fig. 3 and 5×10^6 bone marrow cells in Fig. 2b).

The features of 'high dose' tolerance were different from those of 'low dose' tolerance. Although the tolerant state of the donor animals injected with a 'high dose' was not so complete as that induced with a 'low dose' (Katsura, 1972b), the injection of the tolerogen at 'high dose' seemed to suppress the reactivity of cells in spleen, thymus and bone marrow. Chiller *et al.* (1970, 1971) reported the suppressive effect of deaggregated bovine γ-globulin on the cells of both thymus and bone marrow. In those experiments, a relatively 'high dose' of 2·5 mg was used for the tolerance induction. Thus, it may be concluded that the injection of tolerogen in a 'low dose' causes mainly unresponsiveness of cells in peripheral lymphoid systems and 'high dose' tolerance involves, in addition, the suppression of both thymus cells and bone marrow cells. The difference in the locality of suppressed cells in relation to the dose of tolerogen may possibly be ascribed to either or both of the two factors; one is the necessity for large amounts of antigen for penetrating into thymus and bone marrow, and the other is the relatively low susceptibility of cells in those tissues.

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