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Studies on B-cell memory

I. GENERATION AND EXHAUSTION OF B-CELL MEMORY BY THYMUS-DEPENDENT ANTIGEN IN T-CELL DEPLETED MICE

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Summary. Mice depleted of T cells by adult thymectomy, X-irradiation and reconstitution with syngeneic bone marrow cells, either untreated or treated with anti-Thy-1 serum and complement, were immunized intensively with alum-precipitated bovine serum albumin (AP-BSA) along with or without bacterial lipopolysaccharide (LPS), but no significant anti-BSA antibody response was detected. Priming of the T-cell depleted mice, however, either by a single injection of AP-BSA plus LPS or by multiple injections of AP-BSA without LPS, resulted in the generation of immunological memory. A single injection of AP-BSA without LPS was ineffective. The memory required the aid of syngeneic T cells to be recalled by the challenge with AP-BSA plus LPS. On the other hand, multiple injections of AP-BSA plus LPS did not cause the generation of memory and the response of these mice to the challenge was lower than that of unprimed control mice. These results suggest that (1) the anti-BSA response is highly dependent on the helper function of T cells, (2) the degree of T-cell requirement for the memory generation is very low, and (3) priming with too much strong stimulation in the absence of functional T cells leads to the suppression or abortion of previously generated immunological memory.

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INTRODUCTION

Many investigations on the immunolgical memory to T-cell dependent antigens have demonstrated that immunological memory can be carried by T cells and also by B cells (Raff, 1969; Chan, Mishell & Mitchell, 1970; Cunningham & Sercarz, 1971; Miller & Sprent, 1971; Takaoki, 1976). In general, immunological memory in the B-cell population develops more slowly than that in the T-cell population. This may imply that B-cell memory is generated under the influence of helper T cells. Some workers have shown, however, that T cells may not be obligatory for the generation of B-cell memory (Roelants & Askonas, 1972; Diamantstein & Blitstein-Willinger, 1974; Schrader, 1975).

In this series of studies, we have investigated the nature of B-cell memory generated in the presence or in the absence of functional T-cells. The present paper concerns the generation of B-cell memory in the absence of functional T cells for an antigen to which

the antibody response is highly dependent on helper T-cell function.

MATERIALS AND METHODS

Mice

Inbred DDD mice of both sexes (supplied from the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University) were used.

Antigens and immunizations

Crystallin bovine serum albumin (BSA) (Miles Laboratories Inc., Kankakee, Illinois) was used throughout these experiments. Alum-precipitated BSA (AP-BSA) was prepared by the addition of 2 volumes of 10% potassium alum to 7 volumes of 0.5% BSA, followed by neutralization with 10% Na_2CO_3 to pH 6·3 (Katsura, 1972). The precipitate was washed three times by centrifugation and resuspended in 0·15 M NaCl to 2·5 mg or 12·5 mg BSA/ml. For immunization of the mice, 0·4 ml of the AP-BSA suspension with or without 10 μg of bacterial lipopolysaccharide (LPS) (lipopolysaccharide B, extracted from *Escherichia coli* 0111:B4, Difco Laboratories, Detroit, Michigan) was injected. When T-cell depleted mice were challenged 2 weeks after the last priming antigen injection, they were supplemented with 10^8 normal syngeneic thymocytes and injected with 100 μg of AP-BSA plus 10 μg of LPS on the same day and 10 days later injected with 500 μg of AP-BSA plus 10 μg of LPS.

Thymectomy

Six week old mice were thymectomized as previously described (Muramatsu, Amagai & Katsura, 1975). At the time of the last bleeding, all the mice were autopsied. No thymic remnants were found in any animals used in these experiments.

Preparation of cell suspensions

Thymus and bone marrow cell suspensions were prepared following the method previously described (Katsura, Kawaguchi & Muramatsu, 1972). Mice of 6 weeks of age served as cell donors. Eagle's minimum essential medium (Nissui Seiyaku Company, Tokyo) was used for suspending and washing cells. Heparin (heparin sodium injection, Takeda Pharmaceutical Co., Osaka) was added to the medium at 5 U/ml for thymus cell preparation to avoid cell aggregation. Cell suspensions (0·5 ml for bone marrow cells and 1·0 ml for the suspension containing thymus cells) were injected intravenously.

Elimination of Thy-1.2-positive cells

Bone marrow cells were treated with appropriately diluted anti-Thy-1.2 antiserum (AKR anti-C3H thymocyte serum) plus complement as previously described (Inaba, Nakano & Muramatsu, 1978).

X-irradiation

Lymphoid cell recipients were given 800 rad whole body irradiation with an X-ray emitter for experimental use (Toshiba Electric Co., Tokyo) 24 h prior to the cell transfer. Mice were placed in a perforated polystyrene box on a turntable. The irradiation was performed at 200 kV and at a target distance 44 cm, with a filter of 0·5 mm Cu and 0·5 mm Al, at the dose rate of 100 rad/min.

TxXB and TxXBθ mice

Thymectomized mice were rested for 3 weeks and irradiated with 800 rad followed immediately by the injection of 10^7 bone marrow cells which were either untreated (TxXB) or treated *in vitro* with anti-Thy-1.2 antiserum plus complement (TxXBθ). These mice were used in experiments after an additional 3 weeks.

Test for antibody

In these experiments, antibody response was assessed by a passive haemagglutination test for serum antibody. The test is convenient for following the kinetics of antibody response of individual mice, and we have confirmed, in a preliminary test, the correlation between the titre of serum antibodies and the number of antibody-forming cells (plaque-forming cells) in the spleen.

Mice were repeately bled, about 0·1 ml per bleeding, *via* the retro-orbital plexus, and the serum specimens were stored at −20° until the end of each experiment when they were assayed simultaneously. Anti-BSA titration was performed by a passive haemagglutination test according to a previously described method (Hosomo & Muramatsu, 1972). Briefly, glutaraldehyde-fixed mouse erythrocytes were conjugated with BSA by means of *bis*-diazotized benzidine to prepare the indicator red cells. The test was performed using a microtitration device. Each depression of the V-shaped bottom in the titration trays contained 0·025 ml in diluted sera and 0·025 ml of indicator red cell suspension. Mercaptoethanol-resistant (MER) anti-

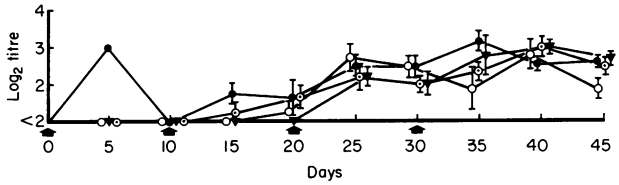

Figure 1. Anti-BSA antibody response of TxXB mice to four injections of antigens at intervals of 10 days. TxXB mice received i.p. injections four times (indicated by arrows): twice with 100 μg of AP-BSA + 10 μg of LPS and twice with 500 μg of AP-BSA + 10 μg of LPS (●), twice with 100 μg of AP-BSA and twice with 500 μg of AP-BSA (▾), four times with 10 μg of LPS (⊙), or four times with 0·15 M NaCl (○). Each point refers to the geometric mean ± one SE of total antibody titres of eight to ten mice.

body titres were determined as described previously (Muramatsu *et al.*, 1975).

RESULTS

Inability of TxXB mice to produce anti-BSA antibody

TxXB mice were immunized by four injections of AP-BSA with or without 10 μg of LPS at intervals of 10 days. Control TxXB mice were given either LPS alone or 0·15 M NaCl. Mice were bled every 5 days. Results are shown in Fig. 1. No significant level of anti-BSA antibody was detected in any group of mice throughout the experiment, except for a group of mice 5 days after the first injection of AP-BSA plus LPS. Other than this, apparently positive haemagglutination activities in sera were non-BSA specific, since they were not inhibited by the addition of BSA (1 mg/ml) to the titration medium.

These results indicate that the anti-BSA response is highly T-cell dependent even when aided by LPS, and that our TxXB mice have few helper T cell precursors capable of responding to BSA.

Time course of the generation of B-cell memory in TxXB mice.

TxXB mice were primed with a single injection of 100 μg of AP-BSA with or without LPS and challenged 5, 10, or 34 days later to estimate memory generation. One hour before the challenge, these TxXB mice were given 10^8 syngeneic normal thymocytes as a T-cell source. The challenge was performed twice at an interval of 10 days: 100 μg of AP-BSA plus 10 μg of LPS as primary (1°) challenge and 500 μg of AP-BSA plus 10 μg of LPS as secondary (2°) challenge.

Results are shown in Fig. 2. A memory state was not detected when mice were challenged 5 days after priming (Fig. 2a), but could be detected by the challenge 10 (Fig. 2b) or 34 days (Fig. 2c) after priming. Figure 2b also indicates that LPS was necessary as an adjuvant

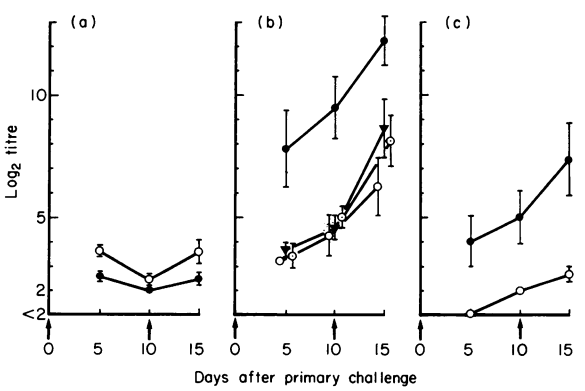

Figure 2. Time course of B-cell memory generation in TxXB mice. TxXB mice given single i.v. injection of 100 μg of AP-BSA + 10 μg of LPS (●), 100 μg of AP-BSA (▾), 10 μg of LPS (⊙), or 0·15 M NaCl (○) were challenged (a) 5 days, (b) 10 days, or (c) 34 days later with i.v. injection of 100 μg of AP-BSA + 10 μg of LPS, followed 10 days later by i.p. injection of 500 μg of AP-BSA + 10 μg of LPS. One hour before the challenge, all mice were given 10^8 syngeneic normal thymocytes. Each point refers to the geometric mean ± one SE of the total antibody titres of three to seven mice.

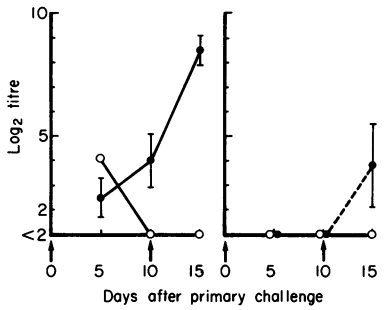

Figure 3. Generation of B-cell memory in TxXBθ mice. TxXBθ mice given i.v. injection of either 100 μg of AP-BSA + 10 μg of LPS (•) or 0·15 M NaCl (○) were challenged 14 days later with i.v. injection of 100 μg of AP-BSA + 10 μg of LPS, followed 10 days later by i.p. injection of 500 μg of AP-BSA + 10 μg of LPS. One hour before the challenge, all mice were given 10^8 syngeneic normal thymocytes. Each point refers to the geometric mean ± one SE of the total (left panel) or 2-mercaptoethanol resistant (right panel) antibody titres of four mice.

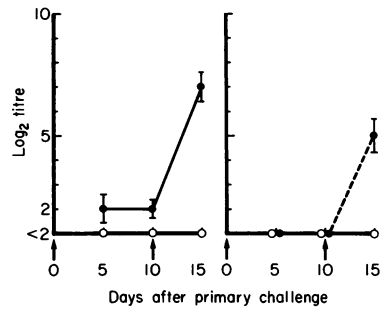

Figure 4. IgG memory generation in TxXBθ mice after four injections of AP-BSA without LPS. TxXBθ mice given four injections of AP-BSA (100 μg twice and 500 μg twice) (•) or 0·15 M NaCl (○) at intervals of 4 days were challenged 14 days later as described in Fig. 3. One hour before the challenge, all mice were given 5×10^7 syngeneic normal thymocytes. Each point refers to the geometric mean ± one SE of the total (left panel) or 2-mercaptoethanol resistant (right panel) antibody titres of six mice.

to generate anti-BSA memory state, since a single injection of AP-BSA without LPS was ineffective.

Figure 3 shows the result of an experiment in which TxXBθ mice were primed with 100 μg of AP-BSA plus 10 μg of LPS and challenged 14 days later. The anti-BSA memory was also seen as in the case of TxXBθ mice.

Effect of multiple pre-injections of antigens in TxXB and TxXBθ mice.

TxXBθ mice were given four injections of AP-BSA without LPS, and challenged 2 weeks after the last injection. They received 10^8 thymocytes before 1° challenge, and 2° challenge was performed 10 days later. Results are shown in Fig. 4. Pre-immunized mice showed an enhanced antibody response to the challenge. This indicates that multiple injections of AP-BSA were sufficient to generate immunological memory, although a single injection of AP-BSA alone was ineffective (Fig. 2b).

In another experiment, of which the results are shown in Fig. 5, TxXB mice (Fig. 5a) and TxXBθ mice (Fig. 5b) were given four injections of AP-BSA plus LPS and challenged with AP-BSA plus LPS soon after the supplementation with thymocytes as in the experiment of Fig. 4. It was found that the multiple injections of AP-BSA with LPS resulted in the reduction of the response to the challenge immunization. Thus, these results suggest that too much strong antigenic stimulation in the absence of functional T cells may cause partial tolerization or abortive exhaustion of B cells.

The anti-BSA antibody response in saline-injected control animals seems to vary among different experiments (compare Figs 3 and 5b). This may be attributable to differences in the experimental conditions. First, the interval of time between the reconstitution of TxX mice with bone marrow cells and the challenge of mice with antigen after priming injections was not always the same. For example, the intervals were about 7 weeks in Fig. 5b and 5 weeks in Fig. 3. Second, the sensitivity of indicator red cells in the passive haemagglutination test to anti-BSA antibodies might not be the same in different experiments according to the time length after preparation and the difference in the lot. Thus we have compared absolute values only within one experiment.

DISCUSSION

These experiments indicate that the anti-BSA antibody response is highly dependent on the helper action of T cells, but the immunological memory which can be recalled in the presence of T cells was established in TxXB and TxXBθ mice. We cannot exclude a possibility that a small number of T cells, radioresistant in the host and/or derived from bone marrow cells injected, may be present in these mice. The finding, however, that they showed no appreciable antibody response even to immunization by multiple injections of AP-BSA plus LPS (Fig. 1), which is highly immunogenic

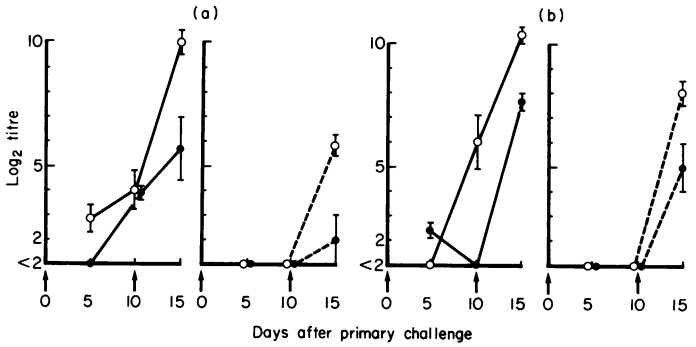

Figure 5. Negative memory in TxXB or TxXBθ mice given four injections of AP-BSA plus LPS. (a) TxXB mice injected twice with 100 μg of AP-BSA + 10 μg of LPS and twice with 500 μg of AP-BSA + 10 μg of LPS (•), or four times with 10 μg of LPS (○) at intervals of 10 days were challenged 14 days later as described in Fig. 3. (b) TxXBθ mice injected twice with 100 μg of AP-BSA + 10 μg of LPS and twice with 500 μg of AP-BSA + 10 μg of LPS (•), or four times with 0·15 M NaCl (○) at intervals of 4 days were challenged 14 days later as described in Fig. 3. Each point refers to the geometric mean ± one SE of the total (left panel) or 2-mercaptoethanol resistant (right panel) antibody titres of three to five mice.

for intact mice (data not shown), strongly suggests the deficiency in BSA-specific helper T cell function in our 'T-depleted mice'. Chiller and Weigle (1973) demonstrated that, if aided by LPS, even TxXBθ mice responded well to human gammaglobulin (HGG) which is one of the T-cell dependent antigens. Thus, the effectiveness of LPS in stimulating antibody response of TxXBθ mice seems to differ between BSA and HGG.

Our results essentially conform to the observations of some other investigators. Roelants & Askonas (1972) reported the induction of B-cell memory in TxXB mice for a T-cell dependent antigen (*Maia squinado* haemocyanin). A similar observation was made by Diamantstein & Blitstein-Willinger (1974) in congenitally athymic nude mice employing sheep erythrocytes as antigen. Furthermore, Schrader (1975) demonstrated the generation of hapten-specific B-cell memory in nude mice immunized with hapten-carrier conjugates in a T-cell dependent form. Thus, the helper function of T cells seems to be not always obligatory in the generation of B-cell memory for T-cell dependent antigens.

TxXB mice immunized with a single injection of AP-BSA without LPS showed neither antibody response (Fig. 1) nor memory generation (Fig. 2b). Four serial injections of AP-BSA without LPS, however, were effective in generating IgG memory in TxXBθ mice. Thus, pre-immunized TxXBθ mice showed enhanced production of 2-ME resistant antibody in response to the challenge with AP-BSA plus LPS in the presence of syngeneic thymocytes (Fig. 4). Whether this IgG memory was carried by surface IgG-bearing B cells or not is unclear in our experiments. Several authors have suggested, however, that IgG memory is carried by IgG-bearing cells. Hämmerling, Masuda & McDevitt (1973) indicated that two immunizations of antigen-specific low responder mice with a synthetic polypeptide resulted in clonal expansion to form specific IgG-bearing cells without antibody production. Their argument is that the switch-over from IgM to IgG may occur at the precursor cell levels without T cell help. Okumura, Metzler, Tsu, Herzenberg & Herzenberg (1976) demonstrated that memory cells bearing surface IgG2a of Ig-1b allotype were induced in the allotype-suppressed mouse which was lacking in the specific helper T cells required for the synthesis of Ig-1b allotype immunoglobulin. These cells were confirmed to be those for IgG2a antibody production (Okumura, Julius, Tsu, Herzenberg & Herzenberg, 1976).

Experimental results shown in this paper and also those of several authors (Diamantstein & Blitstein-Willinger, 1974; Schrader, 1975; Hämmerling *et al.*, 1973; Okumura *et al.*, 1976) seem to indicate that IgG memory for T-cell dependent antigens can be generated even in the absence of functional T cells but expression of the memory may be highly T-cell dependent. We also observed in another experiment that the hapten-specific B-cell memory in athymic nude mice induced by the complex of a hapten and a T-cell dependent carrier antigen could not be recalled by the challenge with the complex of the same hapten and a T-cell independent carrier antigen (data not shown). On the other hand, it has recently been reported that IgG memory generated by T-cell dependent antigens

in the presence of T cells can be expressed by the challenge with T-cell independent antigen (Braley-Mullen, 1976; Kimoto, Kishimoto, Noguchi, Watanabe & Yamamura, 1977; Tittle & Rittenberg, 1978). Therefore, it seems that the IgG memory generated in the absence of functional T cells is different from the IgG memory generated in the presence of functional T cells in their dependence of T cells at their expressions. Thus, we attempted to induce B-cell memory by immunization with a T-cell independent antigen and to manifest the memory under T-cell dependent and T-cell independent conditions. The result is reported elsewhere (Hosokawa, 1979).

The B-cell memory, which was generated in T-cell depleted mice by a single injection of AP-BSA plus LPS, persisted for 1 month at least without further antigenic stimulations (Fig. 2c). Multiple antigenic stimulations with AP-BSA plus LPS, however, resulted in the suppression of BSA-reactive B cells (Fig. 5), though such an immunization schedule was usually effective in inducing both strong antibody response and memory state in intact mice. This seems relevant to our previous observation (Muramatsu *et al.*, 1975) that TxXB mice immunized simultaneously with the mixture of 5 mg of deaggregated BSA and 100 μg of AP-BSA plus 10 μg of LPS became tolerant to BSA whereas normal and TxXBT mice (TxXB mice supplemented with thymocytes) were positively primed by this immunization. These results may suggest that too much strong antigenic stimulation with LPS in the absence of T cells induces immunological tolerance in B cells. Nakashima *et al.* (Nakashima, Nagase, Yokochi, Kojima, Ohta & Kato, 1976) reported that spleen cells from mice which had been stimulated with sheep erythrocytes (SRBC) 14 days previously and with capsular polysaccharide of *Klebsiellar pneumoniae* (CPS-K) 7 days thereafter, showed reduced antibody response to SRBC *in vitro* in comparison with spleen cells from mice not receiving CPS-K. The reduction may be ascribed to the stimulation with CPS-K. Though CPS-K is known to be a strong adjuvant for antibody responses (Nakashima, 1972) as well as a polyclonal B-cell activator (Nakashima & Kato, 1974), injection of CPS-K without antigen may drive B cells and B-memory cells into abortive differentiation and exhaustion even in the presence of helper T cells. In our experiments, B cells and B-memory cells may be abortively exhausted by multiple injections of LPS along with antigen. This seems likely to be attributable to the absence of functional T cells.

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