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Cellular and Humoral Immune Responses in Mice

II. EFFECT OF INTRAPERITONEAL OR SUBCUTANEOUS INJECTION OF CARRIER ON ANTI-HAPTEN ANTIBODY AND DELAYED HYPERSENSITIVITY RESPONSES

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Summary. The subcutaneous (s.c.) injection of sheep red blood cells (SRBC) without adjuvant into mice preferentially induced delayed hypersensitivity (DH) reaction, as measured by footpad swelling, while intraperitoneal (i.p.) injection of the antigen exclusively induced a humoral antibody response, as measured by the haemagglutinin test. Under these conditions, the properties of the helper activities of thymus-derived (T) cells for humoral responses were examined, in association with the features of the DH response, by measuring the anti-hapten and anti-carrier antibody responses after a booster injection of trinitrophenylated (TNP) SRBC and by changing the combination of doses and injection routes of the carrier and the hapten–carrier conjugates.

When mice were presensitized with i.p. injections of SRBC and boosted with i.p. injections of TNP–SRBC, the anti-TNP antibody production was maximally enhanced by presensitization with a low dose of SRBC, and gradually abolished with higher doses of SRBC for pre-sensitization. In the latter case, anti-SRBC antibody production was increased with increasing doses of SRBC. When mice were presensitized with s.c. injections of SRBC and boosted with i.p. injections of TNP–SRBC, enhancement of the anti-hapten antibody production occurred with presensitization to a very low dose of SRBC, and the enhancement was not lessened by presensitization with higher doses of SRBC. In the latter, the titres of anti-SRBC antibody production were relatively low. DH was induced only when the antigen was injected via the s.c. route, and it was augmented with increasing dose of SRBC or TNP–SRBC. However, the development of DH was suppressed by i.p. injections of higher doses of SRBC or TNP–SRBC within 3 days after the s.c. injection of either antigen. DH reactivities measured in various sensitization conditions showed no correlation with the helper activities, estimated by anti-hapten antibody production. Concerning the class distribution of anti-hapten antibody, IgM or IgG predominance was largely influenced by the dose and the time of sensitization with the hapten–carrier conjugate and was not appreciably influenced by the carrier preimmunization.

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From these results, we are tempted to speculate that helper T cells stimulated by SRBC injection are common to IgM anti-SRBC, IgG anti-SRBC, IgM anti-TNP and IgG anti-TNP antibody responses induced by the booster injection of TNP–SRBC, and that the helper T cells are different from DH-related T cells and are present separately before the primary immunization. Apart from the speculation, the present results revealed some characteristics of antigen sensitization via s.c. and i.p. routes.

INTRODUCTION

Delayed hypersensitivity (DH) and antibody production specific for a certain antigen can be elicited separately. Which type of immune response predominates is known to depend on various conditions of antigen injection, e.g. the chemical nature of antigens, doses of antigens, routes of antigen administration, presence or absence of adjuvant, etc. For example, we showed previously that sheep red blood cells (SRBC) injected into mice intraperitoneally (i.p.) in the form of a saline suspension exclusively induce humoral antibody responses, while the antigens preferentially induce DH when injected subcutaneously (s.c.) with Freund's complete adjuvant (Tamura, Kurata, Sugimoto and Egashira, 1973).

Antibody production against SRBC or hapten-conjugated SRBC in mice is known to require the co-operation of thymus-derived (T) cells and bone marrow-derived (B) cells (Claman, Chaperon and Triplett, 1966; Michel and Miller, 1968; Kettman and Dutton, 1970). Some reports on the anti-hapten antibody production demonstrate that prior injection of a carrier antigen strikingly enhances the subsequent response to a hapten coupled to the same carrier (Rajewsky, Schirrmacher, Nase and Jerne, 1969; Katz, Paul, Goidl and Benacerraf, 1970; Kettman and Dutton, 1971) and the carrier-primed cells which enhance the hapten response are thymus-derived (Raff, 1970). On the other hand, T cells are also considered to play an important role in DH of mice as the antigen-reactive cells (Eidinger and Ackerman, 1971). Under the circumstances, an investigation of the detailed properties of T cells involved in cell-mediated and humoral antibody responses would be necessary to elucidate the cell interrelation between both responses.

In the present paper, we established the experimental conditions for DH or humoral response to SRBC to be preferentially induced in mice by s.c. or i.p. injection of SRBC, respectively, with no adjuvant. By using such an experimental system, we further investigated the helper activity of T cells for antibody production and the DH reactivity in mice presensitized with carrier SRBC and sensitized with TNP–SRBC under conditions which involved varying the combination of doses and injection routes of the carrier and the hapten–carrier conjugates. The results obtained provide several important clues for elucidating the lineage, antigen reactivity and differentiation process of the functionally different T cells which play crucial roles in cellular and humoral immune responses.

MATERIALS AND METHODS

Mice

Six to 9-week-old female ddY/S mice were used in all experiments.

Antigens

Commercial SRBC and horse red blood cells (HRBC) stored at 4° in Alsever's solution were washed three times with saline immediately before use.

Preparation of hapten-conjugated RBC

Lightly 2,4,6-trinitrophenyl (TNP) substituted HRBC were used as the antigen in the titration of anti-TNP antibody and were prepared as described by Rittenberg and Pratt (1969). Heavily TNP-substituted SRBC were used as the immunogen and were prepared by the method of Kettman and Dutton (1970).

Immunization

Mice received i.p. or s.c. injections of various doses of SRBC or TNP–SRBC suspended in saline. In the case of the i.p. injections, various doses of the antigen suspension were given in a volume of 100 or 200 μl. For the s.c. injections, the antigens were injected into the lumbar and nuchal regions of each mouse in a volume of 50 or 100 μl.

Antibody assays

Haemagglutinin antibody titres were determined in the sera obtained from mice immediately after measuring DH reactions. The sera were heated at 56° for 30 minutes prior to titration. Haemagglutinin titration was performed with a microtitre set as described previously (Tamura *et al.*, 1973). In order to separate 2-mercaptoethanol-resistant (MER) antibodies from 2-mercaptoethanol-sensitive (MES) ones, sera were diluted with gelatine–veronal-buffered saline containing 0·2 M 2-mercaptoethanol and were incubated at 37° for 30 minutes before addition of antigens. Six-fold dilutions were used initially and serial two-fold dilutions were subsequently made. The number of the last well of the microplate showing visible agglutination was recorded as $\log_2$ titres. Titres of the sera in which the first well did not exhibit a positive reaction were given the value of '0'.

Footpad test

SRBC-specific DH reactions were elicited in mice by injecting 25 μl of 10 per cent SRBC in saline into the right-hind footpad of each animals and 25 μl of saline into the left footpad as a control. DH reactions were determined by measuring the specific increase of footpad thickness at 24 hours after the injection of the eliciting antigen, as described previously (Tamura *et al.*, 1973).

RESULTS

PREFERENTIAL INDUCTION OF HUMORAL ANTIBODY RESPONSE OR DH RESPONSE BY i.p. OR s.c. INJECTION OF SRBC–SALINE SUSPENSION

Kinetics of antibody and DH responses was investigated in mice which had received i.p. or s.c. injections of immunogenic doses of SRBC (2×10^8) without adjuvant (Fig. 1). As shown in Fig. 1a, mice which had received i.p. injections of SRBC did not show any DH throughout the period. On the other hand, DH was induced by s.c. injections of SRBC without adjuvant. DH appeared first on day 3, reached a maximum level on day 4 and thereafter decreased gradually. In the group of i.p.-injected animals, MES antibody

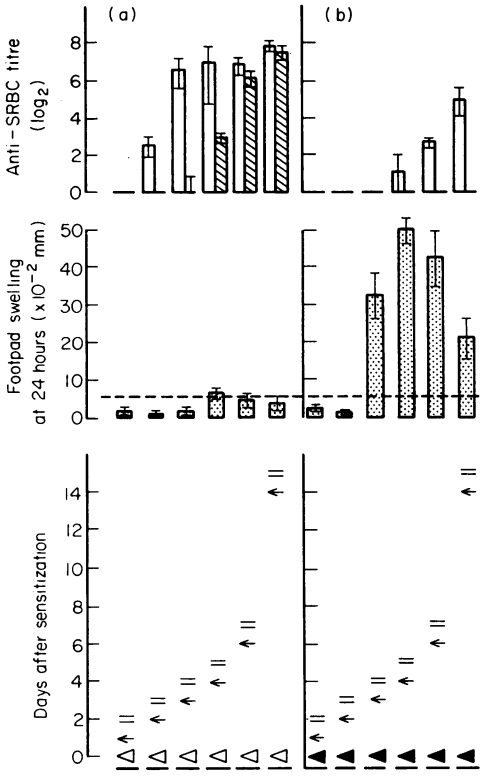

FIG. 1. Kinetics of SRBC-specific delayed hypersensitivity (DH) and humoral antibody responses after sensitization with i.p. (△) or s.c. (▲) injections of 2×10^8 SRBC in saline. The eliciting antigen for DH reactions was injected into the footpad at the time indicated with the arrow (←). Twenty-four hours later, footpad swelling and haemagglutinin titre were determined (=). For details of the experimental methods see the Materials and Methods section. Stippled columns and the dotted line represent the footpad swelling at 24 hours and the upper limit of standard deviation of footpad swelling in control animals which had been injected with saline in several experiments, respectively. Open columns represent the total haemagglutinin antibody titres and hatched columns represent the 2-mercapto-ethanol-resistant (7S) haemagglutinin antibody titres. Each value represents the mean of five to six mice and the bars indicate the upper and lower limits of the standard error. (a) Kinetics of the immune responses after i.p. injection and (b) after s.c. injection of SRBC.

(IgM) production began to increase on day 3, reached a maximum on day 4, and was followed by the production of MER antibodies (IgG). In the group of s.c.-injected animals, only slight IgM production occurred on day 5, which continued to increase slowly, and no IgG production was detected.

The effects of i.p. or s.c. injections of various doses of SRBC (2×10^5–2×10^9) on the induction of DH and antibody responses were investigated on day 6 after immunization. Fig. 2a shows that no appreciable DH was observed at any antigen dose in animals which had received i.p. injections of SRBC, while serum antibody production occurred with 2×10^6 SRBC and reached a plateau with 2×10^8 SRBC. On day 6 after immunization, only IgM production was detected with lower doses of antigen (2×10^6–2×10^7) and both IgM and IgG production were seen with higher doses of SRBC (2×10^8–2×10^9) (Fig. 2a). On the other hand, DH reactions were induced with s.c. injections of 2×10^6 SRBC and augmented with higher doses, whereas serum antibody was barely detected with lower

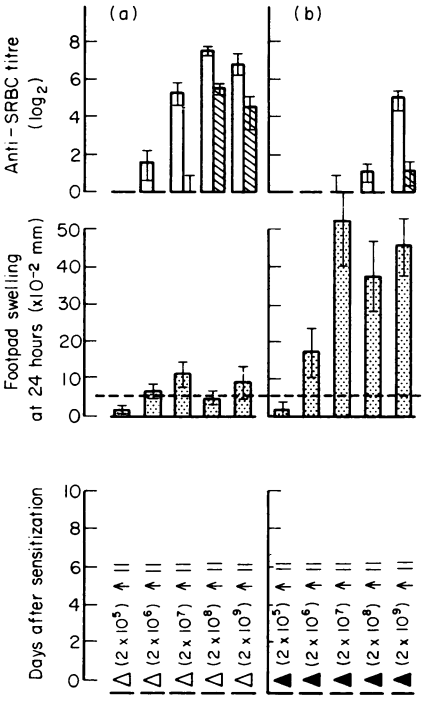

FIG. 2. DH and humoral antibody responses in mice sensitized with i.p. (△) or s.c. (▲) injection of various doses of SRBC. The figures in parentheses represent the doses of SRBC. For other symbols and details, see caption to Fig. 1. (a) Dose dependence of the immune responses induced by i.p. and (b) by s.c. injection of SRBC.

doses (less than 2×10^9 SRBC). These results indicate that an antibody response can be exclusively induced by i.p. injection of 2×10^7 or more SRBC in saline from days 3–5 after sensitization, while DH response can be preferentially induced by s.c. injection of 2×10^6–2×10^8 SRBC in saline from days 3–5 after sensitization.

EFFECT OF i.p. PRE-INJECTION OF CARRIER SRBC ON ANTI-HAPTEN ANTIBODY AND DH RESPONSES

Anti-TNP antibody responses on day 4 or 6 after i.p. injection of TNP–SRBC were investigated in mice which had received i.p. injections of various doses of SRBC 6 days before. Fig. 3a shows that mice that had previously received i.p. injections of various doses of SRBC (2×10^5–2×10^7) produced MES anti-TNP antibody (IgM) on day 6 after injection of 2×10^6 TNP–SRBC, although the anti-hapten antibody was not detected in sera from non-pretreated mice. The maximum enhancement of anti-TNP production was observed in mice pre-sensitized with 2×10^6 SRBC and the increase in dose of SRBC for pre-sensitization brought about the decrease of such an enhancement. On the other hand, anti-SRBC antibody responses were heightened with increase in dose of carrier SRBC. Fig. 3b shows that on day 6 after injection of 2×10^8 TNP–SRBC in non-pretreated mice, considerable amounts of both MES (IgM) and MER (IgG) anti-hapten antibodies

were observed. Pre-sensitization of mice with low doses of SRBC (2×10^5–2×10^6) slightly enhanced anti-hapten antibody responses against 2×10^8 TNP–SRBC, while presensitization with higher doses of SRBC (2×10^8–2×10^9) lessened IgG anti-TNP antibody responses. Under these conditions, higher level of anti-SRBC antibody responses were observed in all cases of SRBC pre-sensitization and IgG anti-SRBC antibody responses

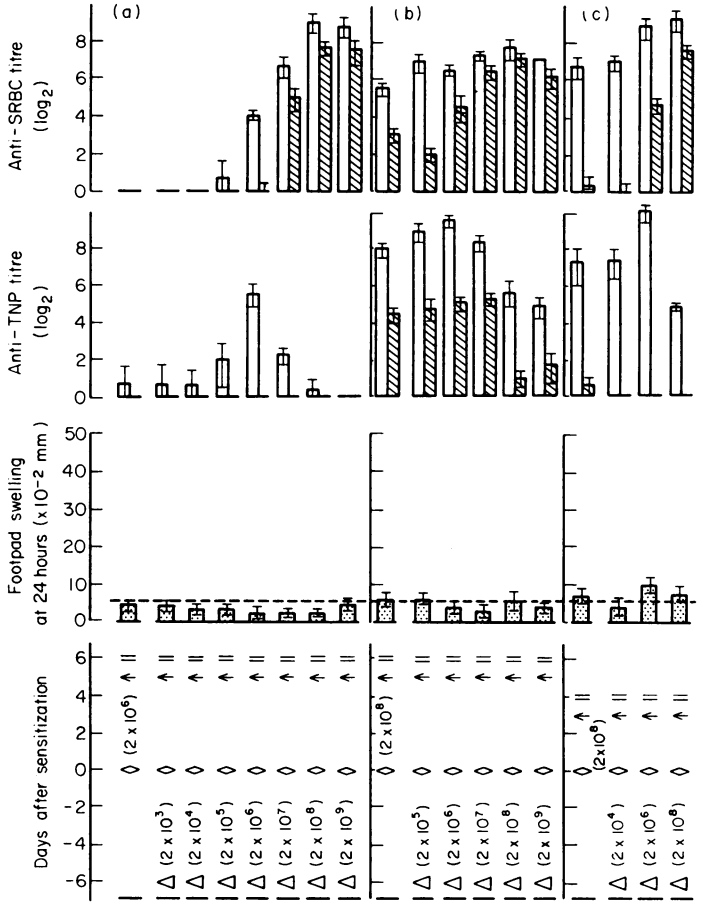

FIG. 3. DH, anti-TNP antibody and anti-SRBC antibody responses in the mice pre-sensitized with i.p. injection of various doses of SRBC (△) on day 6 before i.p. injection of TNP–SRBC (◇). For other symbols and details, see caption to Fig. 1. The figures in parentheses show the doses of SRBC or TNP–SRBC. Each value represents the mean of five mice. (a) Immune responses on day 6 after injection of 2×10^6 TNP–SRBC in normal or pre-sensitized mice; (b) those on day 6 after injection of 2×10^8 TNP–SRBC; (c) immune responses on day 4 after injection of 2×10^8 TNP–SRBC.

were particularly augmented with increases in the dose of SRBC. As shown in Fig. 3c, on day 4 after injection of 2×10^8 TNP–SRBC, enhancement or depression of IgM anti-hapten antibody response was observed when mice had been pre-sensitized with 2×10^6 or 2×10^8 SRBC, respectively. No appreciable DH was observed in mice that had received i.p. injections of SRBC and TNP–SRBC. These results indicate that the antigen dose required for the stimulation of helper T cells was lower than that for the stimulation of B cells for

anti-SRBC antibody production on day 6 after i.p. injection of SRBC (Fig. 2a). The results also showed that the depression of the helper activity in mice receiving i.p. injections of higher doses of SRBC appears to correlate with the appearance of higher titres of anti-SRBC antibody, suggesting that in mice presensitized with higher doses of SRBC, the increased population of SRBC-specific B cells competes with a small population of TNP-specific B cells for co-operation with the common helper T cells. In addition, the immunoglobulin class of anti-hapten antibody and the magnitude of the response in the presensitized mice largely depended on the dose of the hapten–carrier conjugate and on the interval between the time of hapten–carrier injection and the determination point of serum antibody titre (Fig. 3). This result also suggests that the helper T cells involved in IgM and IgG anti-TNP antibody production are common to both, and the class of anti-hapten antibody and the magnitude of the response reflect the differentiation stages of B-cell population after sensitization with the hapten–carrier conjugates.

The kinetics of appearance of carrier-specific helper activity was examined by measuring

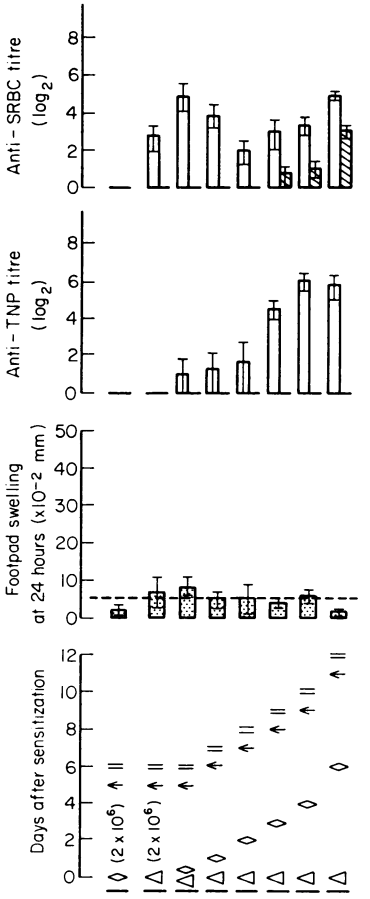

FIG. 4. Kinetics of the development of an enhanced anti-TNP antibody response in the mice presensitized with i.p. injection of 2×10^6 SRBC (△). Mice were then sensitized with i.p. injection of 2×10^6 TNP–SRBC (◇) at the indicated points. For other symbols and details, see caption to Fig. 1.

anti-TNP antibody titres in mice that had received i.p. injections of 2×10^6 TNP–SRBC on days 0, 1, 2, 3, 4 and 6 after an i.p. injection of 2×10^6 SRBC. As shown in Fig. 4, presensitization with an i.p. injection of 2×10^6 SRBC exerted an enhancing effect on the IgM anti-TNP antibody response against 2×10^6 TNP–SRBC from day 3 after presensitization. This result indicates that helper T cells become functional from day 3 after an i.p. injection of SRBC.

EFFECTS OF S.C. PRE-INJECTION OF CARRIER SRBC ON ANTI-HAPTEN ANTIBODY AND DH RESPONSES

Anti-TNP antibody responses on day 4 or 6 after i.p. injections of 2×10^6 or 2×10^8 of TNP–SRBC were investigated in mice that had received s.c. injections of various doses

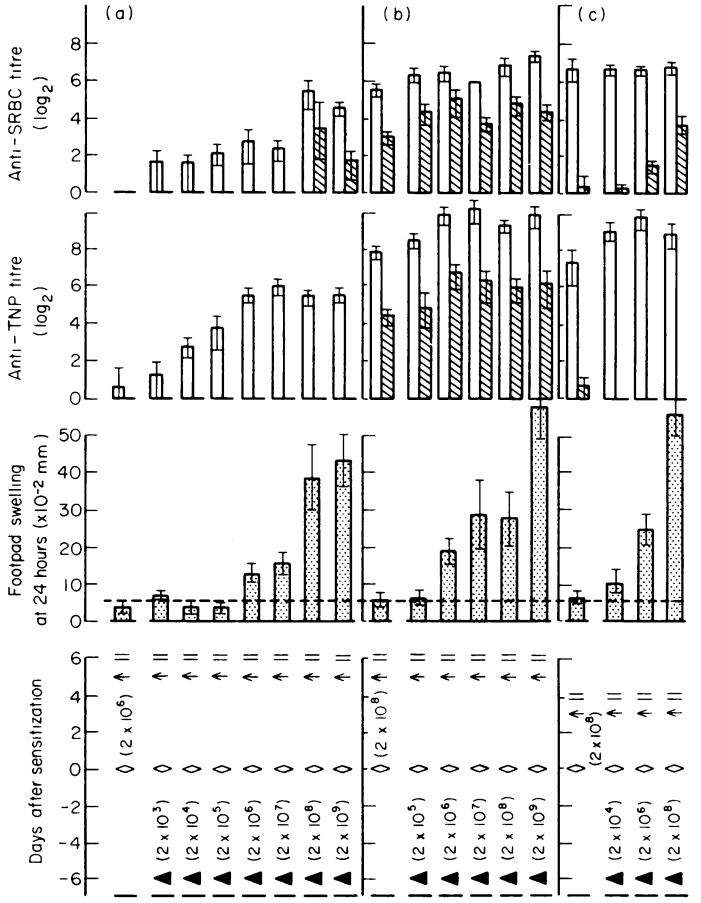

FIG. 5. DH, anti-TNP antibody and anti-SRBC antibody responses in the mice pre-sensitized with s.c. injections of various doses of SRBC (▲). The booster i.p. injections of 2×10^6 or 2×10^8 TNP–SRBC (◇) were performed on day 6 after s.c. presensitization. For other symbols and details, see caption to Fig. 1. (a) Immune responses on day 6 after i.p. injection of 2×10^6 TNP–SRBC in the normal or presensitized mice; (b) those on day 6 after injection of 2×10^8 TNP–SRBC; (c) immune responses on day 4 after injection of 2×10^8 TNP–SRBC.

of SRBC (2×10^3–2×10^9) 6 days before the i.p. injection (Fig. 5). Enhancement of IgM anti-TNP antibody response against 2×10^6 TNP–SRBC occurred significantly in mice presensitized with 2×10^4 SRBC, and reached a maximum level with 2×10^6 SRBC which was maintained with higher doses of SRBC (Fig. 5a). Moreover, on day 6 after an i.p. injection of 2×10^8 TNP–SRBC, appreciable enhancement of IgG anti-hapten antibody production was observed in mice presensitized with 2×10^6 or more SRBC (Fig. 5b), and on day 4, the enhancement of IgM anti-hapten antibody response was observed in presensitized mice (Fig. 5c). On the other hand, the development of DH reaction clearly depended on the doses of SRBC for presensitization, regardless of the doses of the post-injection of TNP–SRBC via the i.p. route. These results indicate that the antigen dose required for the stimulation of helper T cells was lower than the antigen dose necessary for the development of DH, and that the mode of the dose-dependent response of the helper activity was different from that of DH. Furthermore, the helper T cells in mice that

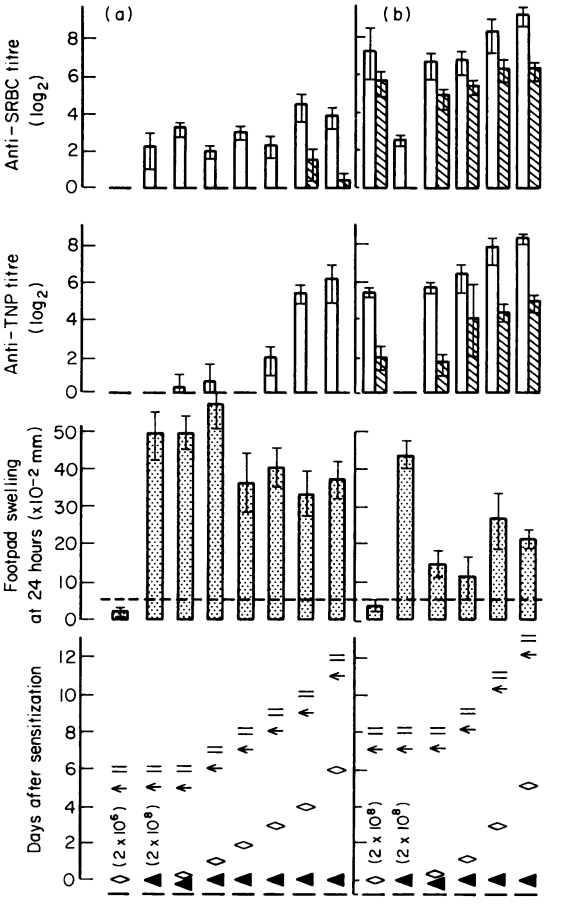

FIG. 6. Kinetics of the development of an enhanced anti-TNP antibody response in the mice presensitized with s.c. injection of 2×10^8 SRBC (▲). The booster i.p. injections of 2×10^6 or 2×10^8 TNP–SRBC (◇) were performed at the indicated points. For other symbols, see caption to Fig. 1. (a) Immune responses on day 6 after injection of 2×10^6 TNP–SRBC; (b) those on day 8 after injection of 2×10^8 TNP–SRBC.

had received s.c. injections of SRBC were developed with lower doses than those that had received i.p. injections (Fig. 3a). It is also noteworthy that the depression of the helper activity measured by anti-TNP antibody production in mice receiving i.p. pre-injections of higher doses of SRBC (Fig. 3) was not observed in the case of s.c. pre-injection. These results also show that enhancement of IgM anti-TNP antibody production in the carrier-presensitized mice occurred when mice were sensitized with relatively low dose of TNP–SRBC (2×10^6) or when mice were killed on relatively early days (day 4) after sensitization with high doses of TNP–SRBC (2×10^8), while enhanced IgG anti-hapten antibody production occurred when presensitized mice were sensitized with the high dose of TNP–SRBC (2×10^8) via the i.p. route and were killed on relatively late days (day 6). From these results, it is possible to suppose that the class of enhanced anti-hapten antibody depends on the dose of TNP–SRBC and on the time after TNP–SRBC sensitization, and that the helper T cells induced by s.c. pre-injection of carrier SRBC act in both IgM and IgG anti-hapten antibody responses, as described in the preceding section.

The time of appearance of carrier-specific helper activity was examined in the mice that had received i.p. injections of 2×10^6 or 2×10^8 TNP–SRBC on days 0, 1, 2, 3, 4, 5 and 6 after s.c. injection of 2×10^8 SRBC (Fig. 6). Enhancement of IgM or IgG anti-TNP antibody responses to 2×10^6 or 2×10^8 TNP–SRBC respectively began to occur on day 3 after s.c. injection of 2×10^8 SRBC (Fig. 6a, b). The timing of the enhancement was correlated with that of DH induced by s.c. injection of SRBC, as described before (Fig. 1b).

In addition, Fig. 6 shows that the development of DH reactions induced by s.c. injection of 2×10^8 SRBC was not influenced by i.p. injection of 2×10^6 TNP–SRBC, but considerably suppressed by i.p. injection of 2×10^8 TNP–SRBC from day 0 to day 2 after s.c. injection of SRBC. These results imply that if antigen-mediated T- and B-cell interaction for antibody production occurs mainly within 3 days after sensitization, development of T cells associated with DH is suppressed.

ANTI-HAPTEN ANTIBODY RESPONSES AGAINST TNP-SRBC INJECTED S.C. IN THE PRESENSITIZED MICE

Anti-TNP antibody responses on day 6 after s.c. injection of 2×10^8 TNP–SRBC were investigated in mice presensitized with i.p. or s.c. injections of varying doses of SRBC 6 days before. As shown in Fig. 7, s.c. injection of TNP–SRBC did not induce anti-TNP antibody responses in mice presensitized with either i.p. or s.c. injections of various doses of SRBC (2×10^5–2×10^9). DH, which was expected to be induced by s.c. injection of TNP–SRBC, was markedly suppressed by the previous i.p. injections of higher doses of carrier SRBC (2×10^7–2×10^9) (Fig. 7a), while it was augmented by s.c. pre-injections of such doses of SRBC (Fig. 7b). These results suggest that s.c. injection of the antigen markedly stimulates both the DH-associated T cells and the helper T cells, but hardly stimulates B cells for antibody production.

EFFECT OF SIMULTANEOUS i.p. AND S.C. PRE-INJECTION OF CARRIER SRBC ON ANTI-HAPTEN ANTIBODY AND DH RESPONSES

Anti-TNP antibody responses on day 6 after i.p. injections of 2×10^6 TNP–SRBC were

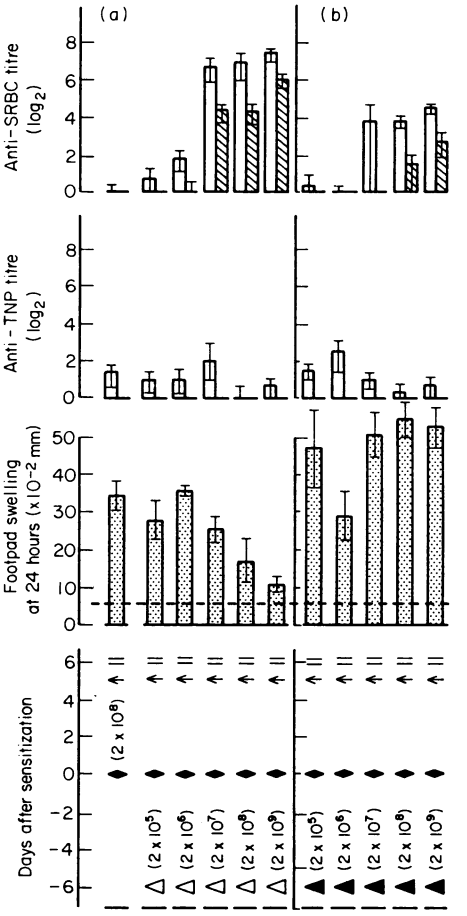

FIG. 7. DH, anti-TNP antibody and anti-SRBC antibody response in mice presensitized with i.p. or s.c. injections of various doses of SRBC on day 6 before s.c. injection of 2×10^8 TNP–SRBC (◆). For other symbols and details, see caption to Fig. 1. (a) Immune responses 6 days after s.c. injection of TNP–SRBC in normal or mice presensitized by i.p. injection of SRBC (△) at different doses; (b) immune responses in animals presensitized by s.c. injection of SRBC (▲).

investigated in mice which had received both i.p. injections of various doses of SRBC and s.c. injections of a fixed dose of SRBC (2×10^8) 6 days before TNP–SRBC sensitization. In such presensitized mice, enhanced IgM and anti-TNP antibody production occurred nearly equally at any dose of i.p. administered SRBC (Fig. 8). The mode of enhancement of anti-TNP antibody responses in this group was different from that in mice presensitized with only i.p. or s.c. injections of various doses of SRBC (Figs 3 and 5). On the other hand, DH to be induced by an s.c. injection of 2×10^8 carrier SRBC was gradually lowered by the simultaneous i.p. injection of increasing doses of SRBC and almost abolished by i.p. injection of 2×10^9 SRBC, whereas anti-SRBC antibody responses were heightened with increase in SRBC dose via the i.p. route. From this inverse relationship between humoral and cell-mediated immunity, it is suggested that the development of DH-related T cells occurring at the initial phase of sensitization is suppressed under conditions in which

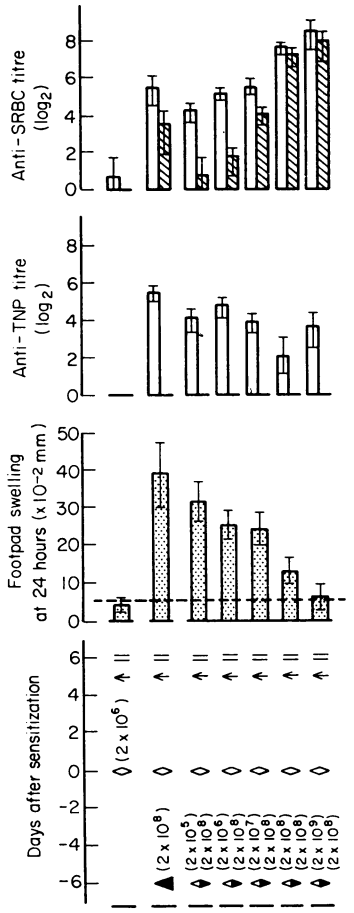

FIG. 8. DH, anti-TNP antibody and anti-SRBC antibody responses in mice presensitized simultaneously with i.p. injections of various doses of SRBC (2×10^5–2×10^9) and s.c. injections of 2×10^8 SRBC (^). Doses of SRBC via the i.p. and s.c. routes are shown in parentheses. The booster i.p. injections of 2×10^6 TNP–SRBC (△) were performed 6 days after the presensitization. For other symbols and details, see caption to Fig. 1.

antigen-mediated T cell–B cell interaction for the antibody production occurs predominantly. In spite of the fact that development of DH-related T cells is suppressed by i.p. injection of higher doses of SRBC, the development of helper T cells for anti-TNP antibody production is not, or only a little, influenced by the same treatments. It seems, therefore, that the development of such helper T cells is independent of that of DH-related T cells.

DISCUSSION

Several methods which can preferentially induce either DH or humoral antibody responses are known. In most of these methods, DH is induced in animals by injecting antigens s.c. in the presence of various adjuvants. In this experiment, DH was preferentially induced by s.c. injection of SRBC with no adjuvant (Figs 1 and 2). The demonstration

that DH or humoral antibody responses is separately induced merely by changing the administration route of the antigen may have practical implications in elucidating the characteristics of administration routes of antigens and the relationship between DH and humoral responses.

The difference between immune responses induced by i.p. injection or s.c. injection of SRBC may be attributed to the difference in distribution of the antigen from inoculation sites into various lymphoid tissues and to the number and the kinds of cells involved in the immune responses in each lymphoid tissue. The primary lymphoid organs stimulated by s.c. injection of SRBC and responsible for the development of DH must be lymph nodes draining the sensitization sites. On the other hand, the main lymphoid organ stimulated by i.p. injection of SRBC and involved in the antibody production must be spleen. In both DH and antibody production against various kinds of antigen, thymus-derived lymphocytes have been known to play important roles. It has been reported that in the lymph nodes the percentage of T cells was high and that of B cells was low, while an inverse relationship between percentages of T cells and B cells existed in the spleen (Raff and Owen, 1971; Rabellino and Owen, 1971). Moreover, there has been increasing evidence to suggest that there are two subpopulations of T cells with different functional and physical properties, that is, one of the cells (T_1) is present in high concentration in thymus and spleen and the other cell (T_2) is present in high concentration in lymph node, blood and thoracic duct (Raff and Cantor, 1971). The functional significances of these cells associated with DH and humoral responses are not well understood.

The present experiments showed that, in the case of i.p. injections, prior priming with a relatively low carrier antigen dose brought about an enhanced anti-hapten antibody response and priming with a high antigen dose resulted in diminution of the anti-hapten antibody response (Fig. 3). These results are consistent with those reported by Kappler, Hoffman and Dutton (1971), indicating that the anti-TNP antibody production *in vitro* by spleen cells was maximal when mice were primed with a suboptimal dose of the carrier SRBC, while priming with an optimal dose of SRBC for PFC production gave less helper activity. Many explanations are possible for the mechanism of diminution of helper activity induced by i.p. pre-injection of higher doses of SRBC. Tada, Okumura and Taniguchi (1972) reported that pre-immunization of rat with *Ascaris* antigen (Asc) in Freund's complete adjuvant resulted in suppression of anti-DNP IgE antibody on stimulation with DNP–Asc and suggested that over-proliferation of carrier-specific T cells may result in suppression of anti-hapten antibody. Takatsu, Hamaoka and Kitagawa (1973) reported that the anti-carrier antibody generated by carrier pre-immunization suppresses the subsequent anti-hapten antibody response in immunized animals. However, it is reasonable to suppose from the present results that in mice presensitized with higher doses of SRBC via the i.p. route, SRBC-specific B cells must be stimulated and markedly increased in number as compared with TNP-specific B cells. Under these circumstances, the apparent decrease of helper activity estimated by anti-hapten antibody production would occur as a result of competition between an increased population of SRBC-specific B cells and a very small population of TNP-specific B cells for the common carrier-specific helper T cells after i.p. injection of TNP–SRBC (Fig. 3). This notion was consistent with that of Falkoff and Kettman (1972).

The present experiments also showed that the mode of development of anti-hapten antibody responses in mice presensitized with an s.c. injection of SRBC was different from that obtained with an i.p. injection, that is, s.c. pre-injection with higher doses of SRBC

ranging from 2×10^7 to 2×10^9 caused a pronounced enhancement of anti-hapten antibody response (Fig. 5). The difference in the helper T-cell activity between i.p. and s.c. pre-injection of SRBC appears to depend on whether SRBC-specific B cells are highly stimulated by SRBC injection via either route. The fact that anti-SRBC antibody response was hardly detected within 6 days after s.c. injection of SRBC ranging from 2×10^5 to 2×10^8 (Fig. 2b) suggests that SRBC-specific B cells were hardly stimulated in the earlier days after s.c. injection of SRBC. In addition, when TNP–SRBC were injected s.c. into mice presensitized with i.p. or s.c. injections of SRBC, anti-TNP antibody response was hardly induced (Fig. 7). This also shows that antigen administration via a s.c. route results in stimulation of T cells associated with both helper activity and DH, but does not stimulate B cells. Hence, the pronounced enhancement of anti-hapten antibody response must occur in mice presensitized with higher doses of SRBC via the s.c. route and followed by sensitization with TNP–SRBC via the i.p. route (Fig. 5). Under such presensitization conditions, only T-cell populations seem to be stimulated, but SRBC- and TNP-specific B-cell populations seem to remain unchanged in number. The subsequent TNP–SRBC sensitization would render the SRBC- and TNP-specific B cells to develop equally and bring about pronounced anti-SRBC and anti-TNP antibody productions with the aid of maximally activated helper T cells.

Furthermore, it was shown that in mice having previously received both i.p. and s.c. injections of SRBC, anti-hapten antibody response did not disappear after i.p. injection of TNP–SRBC (Fig. 8), although anti-hapten antibody responses were abolished in the animals given only i.p. pre-injections of higher doses of SRBC (Fig. 3). This finding cannot simply be understood to be the result of differences in population between SRBC- and TNP-specific B cells. Probably some other mechanisms exist; for example, the helper T cells stimulated by s.c. pre-injection of SRBC might recirculate from the lymph nodes to the spleen after stimulation of an i.p. injection of TNP–SRBC.

In addition to the possibilities that either T cells or T and B cells were preferentially sensitized, depending on the difference in antigen administration via s.c. and i.p. routes, the present data also showed that the nature of the T-cell population stimulated by s.c. injection of SRBC seemed to be different from that stimulated by i.p. injection. The antigen dose required for stimulation of the helper T cells resulting from s.c. injection of SRBC is much lower than that resulting from i.p. injection (Figs 5 and 7). Moreover, DH was induced by s.c. injection of SRBC, but not by i.p. injection. These findings may be correlated with the difference in distribution of T_1 and T_2 cells in lymph nodes or spleen, as reported by Raff and Cantor (1971).

On the class distribution of anti-hapten antibodies between IgM and IgG, the results showed that it depended on doses of TNP–SRBC and on the time after TNP–SRBC sensitization, and was not influenced by the presensitization of SRBC (Figs 3 and 5). This fact suggests that the class of the emerging antibodies is determined by the antibody-forming cell precursors and that the common helper T cells may act in both IgM and IgG anti-hapten antibody responses. Katz *et al.* (1970) and Schirrmacher and Rajewsky (1970) also reported that the class distribution of anti-hapten antibody between $\gamma 1$ and $\gamma 2$ in the guinea-pig, or IgM and IgG in the rabbit, depended on the time and procedure of priming with hapten–primary carrier conjugates, while it was not appreciably influenced by supplemental immunization with the secondary carrier and booster injection of hapten–secondary carrier conjugates. On the other hand, Kishimoto and Ishizaka (1973) showed evidence that carrier-specific helper cells for the IgE antibody response in the rabbits

were different from those for IgG/IgM antibody, indicating that the carrier-specific helper T cells are heterogeneous. Further experiments to elucidate the common properties of carrier-specific helper T cells involved in IgM and IgG antibody production would need to be conducted.

There appear to be several possible explanations to account for the relationship of functionally different T cells playing roles in cell-mediated immunity and in antibody formation. One of these is that the same T cells could perform both functions, as suggested by Kettman and Dutton (1971). Another explanation is that different immunological activities may be exhibited as the T cells pass through the stages of differentiation (Gordon and Yu, 1973). A third one is that two cells which are derived from a common precursor and differentiate along different pathways after antigen sensitization may mediate cell-mediated immunity or helper function, as suggested by Gordon and Yu (1973). A fourth possibility is that T cells involved in cell-mediated immunity and in helper activity belong to separate subpopulations of T lymphocytes, as suggested by Segal, Cohen and Feldman (1971). Data presented in this paper showed that both helper activity and DH began to develop on day 3 after s.c. injection of SRBC (Figs 1 and 6), whereas the dose dependence of the development of DH was different from that of the helper activity (Fig. 5), that is, although the helper activity began to increase with 2×10^4 SRBC and reached a plateau level with 2×10^6, DH was first detected with 2×10^6 SRBC and augmented with increase in dose of SRBC. This fact indicates that the development of the helper activity is not affected by that of the DH reaction or *vice versa*, suggesting that the helper T cells are different from the T cells involved in DH. In addition, in mice given both i.p. and s.c. pre-injections of SRBC, the helper activity for anti-hapten antibody production was observed even under the suppression of DH (Fig. 8). This validates the previous possibility that the helper T cells are independent of the T cells involved in DH. Thus, the results described may support the fourth hypothesis.

As mentioned before, DH induced by s.c. injection of 2×10^8 SRBC was markedly lowered when higher doses of SRBC were injected i.p. within 3 days after the s.c. injection, whereas anti-SRBC antibody responses were augmented with increasing doses of SRBC by the i.p. route (Figs 6 and 8). The results may essentially support the hypothesis presented by Parish (1971, 1972) that antigen-mediated cell-to-cell interaction between T and B cells is essential for the induction of antibody formation and for suppression of DH-related T-cell development.

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