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Cellular and humoral immune responses in mice

III. ACCELERATION OF DELAYED HYPERSENSITIVITY RESPONSE BY PRESENSITIZATION WITH SUBOPTIMAL DOSE OF ANTIGEN

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Summary. Delayed hypersensitivity (DH) response in mice induced by subcutaneous (s.c.) injection of optimal dose of sheep red blood cells (SRBC) (10^8) was accelerated by s.c. injection of the antigen of 10^3 or more doses, given 2 or more days earlier. The accelerated response appeared soon after the injection of optimal antigen dose, that is, 1 or 2 days earlier than the response of non-presensitized control. The acceleration was antigen specific. The accelerated response was generally accompanied by an acceleration and/or enhancement of humoral antibody response.

Parallel to the acceleration of DH response, the proliferation of regional lymph node cells in the presensitized mice was induced immediately after the following injection of 10^8 SRBC, 1 day earlier than that of non-presensitized animals.

These results suggest that presensitization of mice with the antigen induces DH-related memory cells which proliferate immediately after the following injection and function as effector cells for DH reactions, and that the development of DH-related memory cells occurs in close relation to that of helper thymus-derived (T) cells for antibody production.

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INTRODUCTION

It is well known that primary immunization of animals not only stimulates the formation of antibody, but also induces an altered state which gives an accelerated, heightened and prolonged antibody response upon reinjection of antigen, known as immunological memory. Some reports on immunological memory have shown that prior injection of a low dose of sheep red blood cells (SRBC) induces IgM memory in the mouse (Sercarz and Byers, 1967; Cunningham, 1969). Others have demonstrated that prior injection of SRBC strikingly enhances the subsequent anti-hapten antibody response to a hapten-coupled SRBC and the carrier SRBC-primed cells which enhance the hapten response are thymus derived (T) cells (Raff, 1970; Kettman and Dutton, 1971; Falkoff and Kettman, 1972). The property of immunological memory for antibody production has been also detected in bone marrow-derived (B) cells (Jacobson, L'Age-Stehr and Herzenberg, 1970; Miller and Cudkowicz, 1972; Roelants and Askonas, 1972) and simultaneously in both T and B cells (Miller and Sprent, 1971; Mitchell *et al.*, 1972; Cunningham and Sercarz, 1972). On the other hand, the existence of immunological memory should also be predictable for a delayed hypersensitivity (DH) response, since T cells are considered to play the important role in DH of mice (Crowle, 1975). Under

these circumstances, an investigation of the detailed properties on the DH-related memory would be necessary to elucidate the relationship between cell-mediated and humoral antibody responses.

In the present paper, attempts were made to clarify the existence of immunological memory for DH response by using the experimental system for preferential induction of DH against SRBC which was established previously (Tamura and Egashira, 1975). The results obtained demonstrate the existence and properties of DH-related memory cells and provide several clues for the relationship between the memory cells for DH and helper T cells for humoral antibody response.

MATERIALS AND METHODS

Mice

Six to 10-week-old female ddY/S mice were used in all experiments.

Antigens

Commercial SRBC, horse red blood cells (HRBC) and chicken red blood cells (CRBC) stored in Alsever's solution were washed three times with phosphate-buffered saline solution (PBS) before use.

Sensitization

Mice were sensitized by subcutaneous (s.c.) injection of SRBC or CRBC in PBS into the nuchal and lumbar regions of each animal in a volume of 50 μl each, as reported previously (Tamura and Egashira, 1975).

Measurement of cellular responses in the regional lymph nodes

The extent of lymph node cell proliferation in response to the s.c. injection of SRBC was measured by the incorporation of tritiated thymidine ([^{3}H]TDR) into DNA of the cells in inguinal and brachial lymph nodes of mice. Five to six mice from each experimental group at various days after sensitization were killed by bleeding from the carotid artery and the inguinal and brachial lymph nodes were excised aseptically. Lymph node cells were suspended in 'enriched-MEM'—Eagle's minimum essential medium enriched with sodium pyruvate, non-essential amino acids and glutamine according to the method of Eagle (1959)—under sterile conditions by gently teasing the lymph nodes with a syringe and tweezers. Tissue fragments were removed by filtering the suspension through a 150 mesh stainless steel screen. After washing the cells by centrifugation, they were resuspended in the culture medium to a concentration of about 1×10^7 cells/ml. 0·5 ml of this suspension was added to each culture tube together with an equal volume of the medium containing 4 μCi/ml [^{3}H]TDR (>10 Ci/mM). The culture tubes were then rotated at 1 revolution/8 min for 60 min at 37°. At the end of the culture period, 1 ml of 10 per cent ice-cold trichloroacetic acid (TCA) and further 6 ml of 5 per cent cold TCA were added to the culture tube and the lymph node cells in the tube were washed three times during 1·5 h with 8 ml of 5 per cent cold TCA. The cell pellet after washing was solubilized with a small amount of INSTA-GEL (Packard Instrument Company) and diluted appropriately in a scintillant consisting of POPOP, PPO and toluene. The content of [^{3}H]TDR in the solution was estimated in a Beckman liquid scintillation spectrometer (Model 205B) and expressed as c.p.m. per node.

Antibody assays

Haemagglutinin titres were determined in the sera obtained from mice on various days immediately after measuring DH reactions. Haemagglutinin titration was performed with a microtitre set. Six-fold dilutions of the sera were used initially and serial two-fold dilutions were subsequently made, as described previously (Tamura *et al.*, 1973).

Footpad swelling test

SRBC-specific DH reactions were elicited in mice by injecting 25 μl of 10 per cent SRBC (5×10^7) in PBS into the right-hind footpad of each animal and 25 μl of PBS into the left footpad as a control. DH reactions were determined by the increase of footpad thickness at 24 h after the injection of the eliciting antigen, as described previously (Tamura *et al.*, 1973).

RESULTS

Acceleration of DH response to optimal antigen dose by presensitization with suboptimal dose of SRBC

Kinetics of DH response after s.c. injection of 2×10^8 SRBC were investigated in the mice which had received s.c. injection of suboptimal dose of SRBC

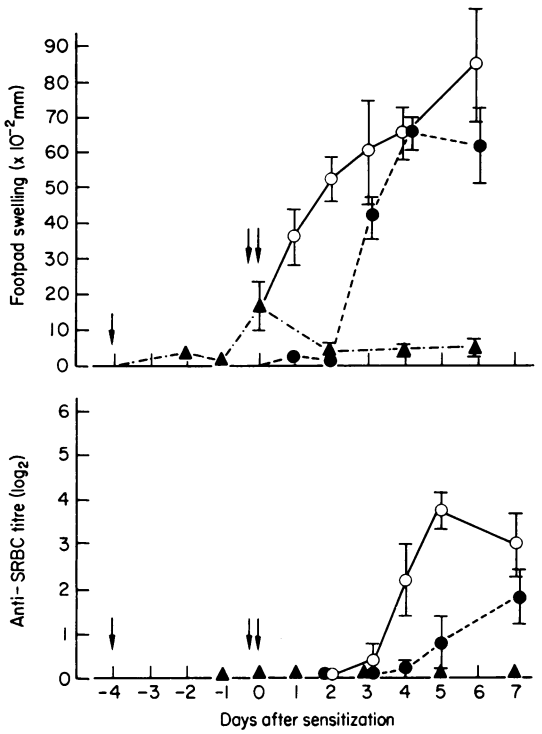

Figure 1. Kinetics of SRBC-specific delayed hypersensitivity (DH) and humoral antibody responses after presensitizing with 2×10^5 SRBC (↓) on day −4 and sensitizing with 2×10^8 SRBC (↓↓) on day 0 (○). Those in the mice sensitized with a single injection of 2×10^5 SRBC on day −4 (▲) or with a single injection of 2×10^8 SRBC on day 0 (●) are also represented. Antigens were administered subcutaneously into the nuchal and lumbar regions of mice for the preferential induction of DH. The footpad swelling at 24 h after injection of eliciting antigen for DH reactions is plotted against the injection time of eliciting antigen, and haemagglutinin titre is plotted against the time when the titre is determined at 24 h after the injection of eliciting antigen. Each value represents the mean of five to six mice and the bars indicate the upper and lower limits of the standard error.

(2×10^5) 4 days earlier. Kinetics of serum haemagglutinin titre were also examined in the animals immediately after measuring the footpad swelling for DH reaction, to investigate the relationship between DH and humoral antibody response (Fig. 1).

No appreciable DH was observed in the mice given a single suboptimal dose of SRBC (2×10^5). In the mice receiving a single injection of 2×10^8 SRBC, DH first appeared on day 3 and reached a maximum level on day 4 and thereafter decreased gradually. When mice were presensitized with 2×10^5 SRBC and sensitized with 2×10^8 SRBC, DH response first appeared on day 1, continued to increase throughout the observed period. Thus, DH response to optimal antigen dose was accelerated by presensitization with suboptimal dose of SRBC and appeared 2 days earlier than in the non-presensitized mice. The acceleration of DH response indicates that immunological memory for DH response is induced by presensitization with the low antigen dose.

The kinetics of humoral antibody response in the same experiment show that antibody production was not observed in the mice that received a single injection of 2×10^5 SRBC, while in the group with a single injection of 2×10^8 SRBC it appeared first on day 5 and thereafter increased gradually. On the other hand, in the mice that received both injections, antibody response appeared one day earlier and at higher titre than that in the group with a single injection of 2×10^8 SRBC. This result shows that humoral antibody response was also accelerated and enhanced when 10^8 SRBC injection was preceded 4 days by an injection of 10^5 SRBC. The acceleration of antibody response was first detected 3 days later than that of DH response.

These results indicate that memory cells for DH response is induced simultaneously with memory cells for humoral antibody response by sensitization with suboptimal dose of SRBC.

Effect of time of presensitization on the accelerated response of DH

The effect of different intervals between presensitization with low dose of SRBC (2×10^5) and sensitization with optimal antigen dose (2×10^8) on the acceleration of DH and antibody responses was investigated in the mice presensitized 1, 2, 3 and 6 days, and 2, 3 and 4 weeks before the following antigen injection.

Fig. 2 shows kinetics of DH and antibody response in the mice presensitized 1, 2, 3 and 6 days earlier. In the mice presensitized with the suboptimal antigen dose 1 day before, normal DH response to the 10^8 SRBC were observed. When the animals were presensitized 2 or more days earlier, an acceleration of DH response was seen. Humoral antibody response was also accelerated when the 10^8 SRBC injection was preceded 2 or more days by presensitization with 10^5 SRBC. In addition, Fig. 3 shows DH and antibody responses in the mice presensitized

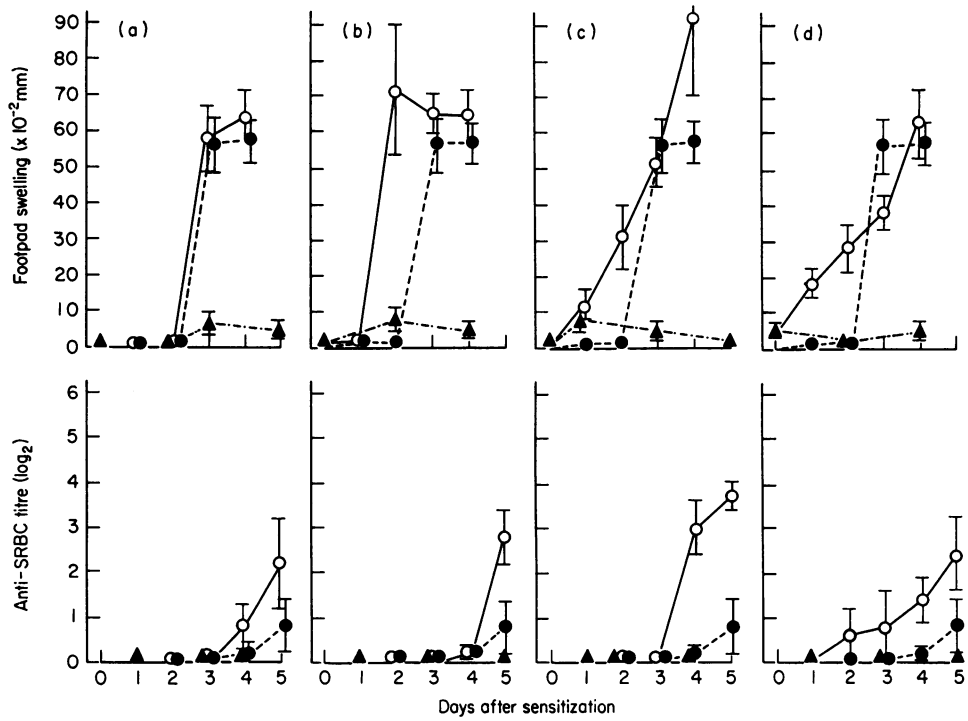

Figure 2. Kinetics of DH and antibody responses in the mice presensitized with 2×10^5 SRBC, (a) 1 day, (b) 2 days, (c) 3 days and (d) 6 days earlier and then sensitized with 2×10^8 SRBC on day 0 (○). Other mice were sensitized with a single injection of 2×10^5 SRBC 1, 2, 3 and 6 days earlier (▲) or with a single injection of 2×10^8 SRBC on day 0 (●). The latter curve is drawn in every figure as a control. For other symbols and details, see caption to Fig. 1.

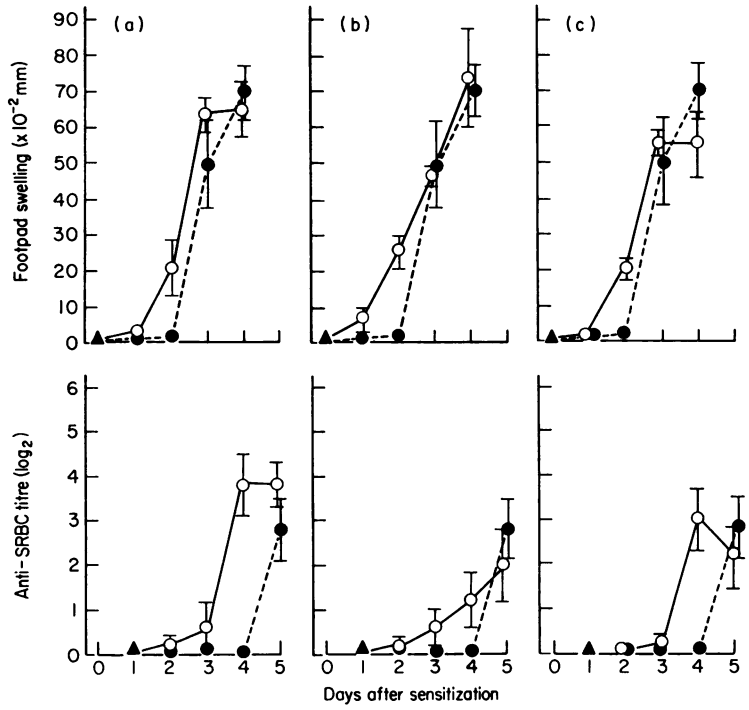

Figure 3. DH and humoral antibody responses to 2×10^8 SRBC in the mice presensitized with 2×10^5 SRBC, (a) 2 weeks, (b) 3 weeks and (c) 4 weeks earlier (○). The same curves representing DH and antibody responses to a single injection of 2×10^8 SRBC (●) are drawn in every figure as a control. The response on day 0 to a single injection of 2×10^5 SRBC given 2, 3 and 4 weeks earlier are represented by (▲). For other symbols and details, see caption to Fig. 1.

2, 3 or 4 weeks earlier. This result indicates that even when 2×10^5 SRBC were injected into the animal 4 weeks earlier, DH and antibody responses to the optimal antigen dose were accelerated.

From these results, it is suggested that the memory effect for DH response appears first on day 2 after sensitization and is maintained for at least 4 weeks and that for humoral antibody also appears almost in the same way.

Effect of different doses of antigen for presensitization on DH response to optimal or suboptimal antigen dose

Effect of different doses of SRBC (ranging from 2×10^8 to 2×10^7) for presensitization on DH response was investigated in the mice which had received various doses of SRBC 4 days earlier and received optimal (2×10^8) or suboptimal (2×10^6) dose of antigen on day 0.

As shown in Fig. 4, in the mice that received a single injection of different doses of SRBC on day −4, DH response from day 0 forth was not detected with 2×10^3 and 2×10^4 SRBC, while in the range of 2×10^5 to 2×10^7 SRBC it was augmented with the increase of antigen dose. When mice received a prior injection of different antigen doses and received the following injection of 2×10^8 SRBC, DH response to 2×10^8 SRBC was accelerated by presensitization with any antigen doses more than 2×10^3 and it exceeded the level of the primary DH response to a single injection of different antigen doses within first 2 days. The difference of the magnitude between accelerated and primary DH responses within first 2 days increased gradually with antigen doses in the range of 2×10^3–2×10^5 SRBC for presensitization, while the difference was lowered in the range of higher antigen doses for presensitization where primary DH response was high. Humoral antibody response, on the other hand, was accelerated and enhanced with increase in dose of SRBC for presensitization.

Fig. 5 shows that DH response to suboptimal

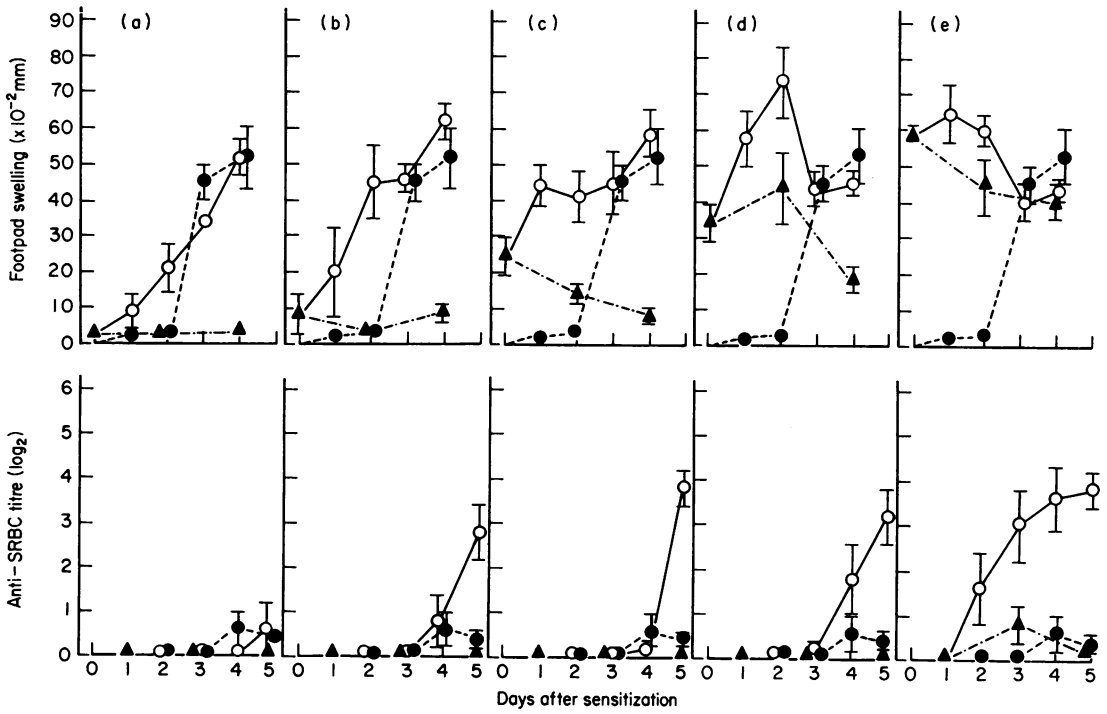

Figure 4. DH and antibody responses to 2×10^8 SRBC in the mice presensitized with: (a) 2×10^3; (b) 2×10^4; (c) 2×10^5; (d) 2×10^6; and (e) 2×10^7 SRBC 4 days earlier (○). The responses in the mice sensitized with a single injection of different doses of SRBC given 4 days earlier (▲) and those in the animals sensitized with a single injection of 2×10^8 SRBC on day 0 (●) are also represented. The latter curve is drawn in every figure as a control. For other symbols and details, see caption to Fig. 1.

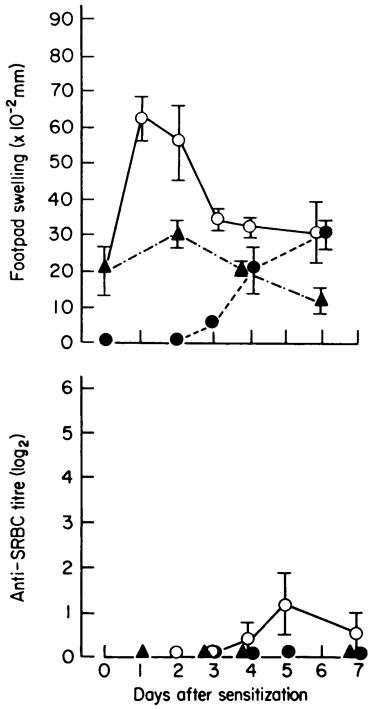

Figure 5. DH and humoral responses after the injection of suboptimal dose of SRBC (2×10^6) in the mice presensitized with 2×10^6 SRBC 4 days earlier (○). Other mice received a single injection of 2×10^6 SRBC 4 days earlier (▲) and on day 0 (●). For other symbols and details, see caption to Fig. 1.

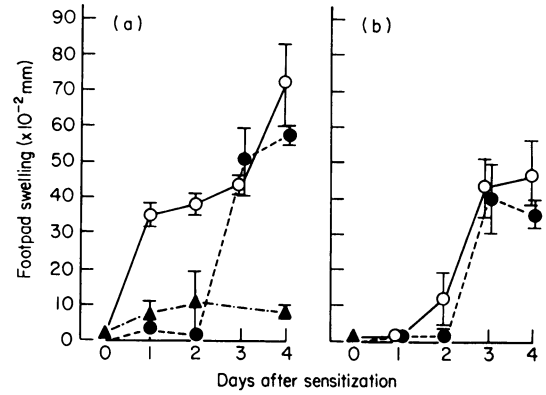

Figure 6. (a) Kinetics of DH response in the mice presentized with 0·001 per cent CRBC 4 days earlier and then sensitized with 1 per cent CRBC (○). DH reaction was elicited by the injection of 0·25 per cent CRBC. The DH responses to a single injection of 1 per cent CRBC given on day 0 (●) and to a single injection of 0·001 per cent CRBC 4 days earlier (▲) are also represented. (b) Kinetics of DH response in the mice presensitized with 0·001 per cent CRBC 4 days earlier and then sensitized with 1 per cent SRBC (10^8) (○). DH reaction was elicited by the injection of 0·25 per cent SRBC. The response to a single injection of 1 per cent SRBC on day 0 (●) and to a single injection of 0·001 per cent CRBC given 4 days earlier (▲) are also shown. For other symbols and details, see caption to Fig. 1.

dose of SRBC (2×10^6) was also accelerated and enhanced in the mice presensitized with 2×10^6 SRBC, and the acceleration and enhancement of DH response was followed by the slight enhancement of antibody response.

These results indicate that DH-related memory is induced by presensitization with the doses of 2×10^3 or more SRBC and stimulated by the second injection of optimal or suboptimal antigen doses to give an acceleration and/or enhancement of DH response, and the memory for antibody production is also induced in a similar manner.

Antigenic specificity of the accelerated response of DH

To test whether the acceleration of DH response was a specific phenomenon, kinetics of DH response were investigated in the animals that were presensitized with 0·001 per cent CRBC 4 days before the sensitization with 2×10^8 SRBC (1 per cent). As shown in Fig. 6a, an accelerated response of DH was observed in an homologous system in which 0·001 per cent CRBC for presensitization and 1 per cent CRBC for the second injection were used. On the other hand, DH response in a heterologous system in which the mice received prior injection of 0·001 per cent CRBC and the second injection of 1 per cent SRBC was not accelerated (Fig. 6b). These results indicate that this phenomenon is specific for antigen.

Cell proliferation in the regional lymph nodes correlated with the acceleration of DH response

In order to obtain some clues to the cellular events on DH-related memory, proliferative response of the regional lymph node cells to 2×10^8 SRBC was investigated in the mice presensitized with 2×10^5 SRBC 4 days earlier. This response was followed by measuring the incorporation of [^{3}H]TDR into DNA of cells obtained from the excised inguinal and brachial lymph nodes (Fig. 7). No appreciable change of the incorporation of [^{3}H]TDR into the lymph node cells was observed throughout the

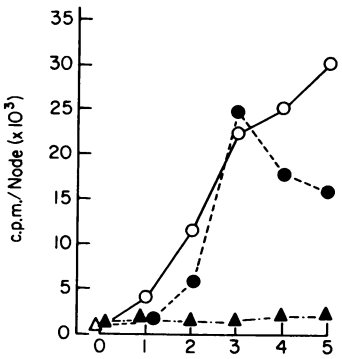

Figure 7. Kinetics of thymidine incorporation into DNA by the inguinal and brachial lymph nodes in the mice presensitized with 2×10^5 SRBC 4 days earlier and then sensitized with 2×10^8 SRBC on day 0 (○). The kinetics in the mice received a single injection of 2×10^5 SRBC 4 days earlier (▲) and a single injection of 2×10^8 SRBC on day 0 (●) are also shown. Open triangle (△) indicates the rate of thymidine incorporation of the lymph node cells in the normal mice.

observed period in the mice that received a single injection of 2×10^5 SRBC on day -4, and the incorporation was a little more than that in normal lymph node cells. The proliferative response of lymph node cells in the animals that received a single injection of 2×10^8 SRBC on day 0 began to increase rapidly from day 2, reached a maximal level on day 3 and thereafter decreased. On the other hand, in the mice that received both injections, the proliferative response began to increase immediately after the second injection of SRBC and continued to increase throughout the period. The onset of this proliferative response was 1 day earlier than that of the non-presensitized mice that received a single injection of 2×10^8 SRBC on day 0. These results show that the proliferative response of the lymph node cells to 2×10^8 SRBC is accelerated by presensitization with 2×10^5 SRBC. The acceleration of cell proliferation in the regional lymph nodes seems to be closely associated with that of DH response.

DISCUSSION

In the present experiments, the existence of DH-related immunological memory was demonstrated by an acceleration of DH to optimal dose of SRBC (10^8) in the mice presensitized with s.c. injection of suboptimal dose of SRBC. The experiments clarified the properties of DH-related immunological memory as described under.

(a) The memory effect was induced by 2×10^5 SRBC given two or more days earlier, even when given 4 weeks earlier (Figs 1, 2 and 3).

(b) The DH-related memory was observed in the mice presensitized with doses of 10^3 or more SRBC (Fig. 4). The memory effect was also detected by an acceleration and enhancement of DH response to suboptimal antigen dose (10^6) in the presensitized mice (Fig. 5).

(c) The memory effect was antigen-specific (Fig. 6). As to the immunological memory for 19S antibody response, Golub (1972) has reported similar results that pretreatment of mice with a suboptimal dose of SRBC (10^6) prepared the animals to make an accelerated 19S PFC response in the spleen to an optimal dose of SRBC (10^8) given two or more days later.

On the difference between primary DH response to a single injection of SRBC and a secondary one to the following antigen injection, the present experiments showed the following.

(a) Primary DH response to optimal dose of SRBC was detected first on day 3 after sensitization, while secondary accelerated DH response was detected soon after the second injection (Fig. 1).

(b) The antigen dose required for the induction of an accelerated and enhanced DH response was lower than that necessary for the induction of primary DH response (Figs 4 and 5).

(c) The primary cellular responses in the regional lymph nodes appeared one day after sensitization, while cells responding to the second injection of antigen began to proliferate within one day (Fig. 7). These results may suggest that the DH-related effector cells that are induced by sensitization with 10^6 or more SRBC are qualitatively different from DH-related memory cells that are induced by sensitization with antigen doses more than 10^3 and result in the accelerated and enhanced DH response upon the following antigen injection. The memory cells would increase in number after primary antigen stimulation and on the second antigen stimulation they would proliferate more rapidly to have a function as effector cells for DH.

As a clue to the relationship between the memory cells for DH and those for antibody production, the present experiments showed that the accelerated DH response was followed by an accelerated and/or enhanced antibody response (Figs 1, 2, 3, 4 and 5).

On the helper cell activity, as a measure of memory cells for antibody production (Raff, 1970; Kettman and Dutton, 1971; Falkoff and Kettman, 1972), we reported previously that anti-hapten antibody response to 2,4,6-trinitrophenylated (TNP-)SRBC was enhanced only in the mice presensitized subcutaneously with 10^3 or more SRBC (Tamura and Egashira, 1975). Fidler, McDaniel, and Golub (1972) showed that pretreatment of mice with 10^6 SRBC given 2 or more days earlier prepares the animals to make an accelerated anti-TNP and anti-SRBC PFC response to 10^8 TNP–SRBC, suggesting that the accelerated response is due to the proliferation of helper T cell population primed with low antigen dose. On the other hand, as shown in the present experiments, DH-related memory cells were induced from day 2 after the presensitization (Fig. 2) and by the injection of 10^3 or more SRBC (Fig. 4). In addition the close correlation between the kinetics of the accelerated cell proliferation (Fig. 7) and that of accelerated DH response (Fig. 1), suggests the possibility that memory cells for DH proliferate rapidly upon the second antigen injection and function as effector cells, to result in the acceleration of DH. Since T cells are known to be involved in the development of DH in mice (Crowle, 1975), it is conceivable that T cells are also involved in DH-related memory. These results may suggest that both DH-related memory T cells and helper T cells not only occur simultaneously, but also have some closely related properties from the similarity of kinetics of sensitization, dose–response relationship and accelerated proliferation of memory cells.

Recently considerable attention has been focused on T-cell heterogeneity (Raff and Cantor, 1971; Stobe and Paul, 1973; Kishimoto and Ishizaka, 1973; Kappler *et al.*, 1974). Several reports demonstrated that T helper cells may not be identical to T effector eells in DH response, although whether they may stem from a common precursor or completely different ones has not yet been clarified. Liew and Parish (1974) reported that T-helper cells in humoral immunity are different from T cells in DH in the recognition of chemically modified flagellin. Elliott and Haskill (1974) distinguished and separated T cells involved in the helper effect from T cells participating in DH by using a rosette technique. These facts may imply that memory T cells for antibody response are not identical to DH-related memory T cells, although both cells have some closely related properties as mentioned above. The investigation of these possibilities must be the subject of future research.

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REFERENCES

Crowle A.J. (1975) Delayed hypersensitivity in the mouse. *Advanc. Immunol.* **20,** 197.

Cunningham A.J. (1969) Studies on the cellular basis of IgM immunological memory. *Immunology,* **16,** 621.

Cunningham A.J. & Sercarz E.E. (1972) The asynchronous development of immunological memory in helper (T) and precursor (B) cell lines. *Europ. J. Immunol.* **1,** 413.

Eagle H. (1959) Amino acid metabolism in mammalian cell culture. *Science,* **130,** 432.

Elliott B.E. & Haskill J.S. (1974) Separation of T effector cells in humoral and cellular immunity. *Nature (Lond.),* **252,** 607.

Falkoff R. & Kettman J. (1972) Differential stimulation of precursor cells and carrier specific thymus-derived cell activity in the *in vitro* response to heterologous erythrocytes in mice. *J. Immunol.* **108,** 54.

Fidler J.M., McDaniel E.M. & Golub E.S. (1972) Regulation of the immune response. III. Effect of the accelerated, response on hapten–carrier responses. *Cell. Immunol.* **4,** 29.

Golub E.S. (1972) Regulation of the immune response. I. Accelerated response induced by suboptimal antigen concentration. *Cell. Immunol.* **3,** 62.

Jacobson E.B., L'Age-Stehr J. & Herzenberg L.A. (1970) Immunological memory in mice. II. Cell interactions in the secondary immune response studied by means of immunological allotype markers. *J. exp. Med.* **131,** 1109.

Kappler J.W., Hunter P.C., Jacobs D. & Lord E. (1974) Functional heterogeneity among the T-derived lymphocytes of the mouse. I. Analysis by adult thymectomy. *J. Immunol.* **113,** 27.

Kettman J. & Dutton R.W. (1971) Radioresistance of the enhancing effect of cells from carrier immunized mice in an *in vitro* primary immune response. *Proc. nat. Acad. Sci. (Wash.),* **68,** 699.

Kishimoto T. & Ishizaka K. (1973) Regulation of antibody response *in vitro*. V. Effect of carrier-specific helper cells

on generation of hapten-specific memory cells of different immunoglobulin classes. *J. Immunol.* **111,** 1.

LIEW F.Y. & PARISH C.R. (1974) Lack of a correlation between cell-mediated immunity to the carrier and the carrier-hapten helper effect. *J. exp. Med.* **139,** 779.

MILLER H.C. & CUDKOWICZ G. (1972) Immunologic memory cells of bone marrow origin. Increased burst size of specific immunocyte precursors. *J. exp. Med.* **135,** 1028.

MILLER J.F.A.P. & SPRENT J. (1971) Cell-to-cell interaction in the immune response. VI. Contribution of thymus-derived cells and antibody-forming cell precursors to immunological memory. *J. exp. Med.* **134,** 66.

MITCHELL G.F., CHAN E.L., NOBLE M.S., WEISSMAN I.L., MISHELL R.I. & HERZENBERG L.A. (1972) Immunological memory in mice. III. Memory to heterologous erythrocytes in both T cell and B cell populations and requirement for T cells in expression of B cell memory. Evidence using immunoglobulin allotype and mouse alloantigen theta markers with congenic mice. *J. exp. Med.* **135,** 165.

RAFF M.C. (1970) Role of thymus-derived-lymphocytes in the secondary humoral immune response in mice. *Nature (Lond.),* **226,** 1257.

RAFF M.C. & CANTOR H. (1971) Subpopulations of thymus cells and thymus-derived lymphocytes. *Progr. Immunol.* **1,** 83.

ROELANTS G.E. & ASKONAS B.A. (1972) Immunological B memory in thymus deprived mice. *Nature: New Biol.* **239,** 63.

STOBE J.D. & PAUL W.E. (1973) Functional heterogeneity of murine lymphoid cells. III. Differential responsiveness of T cells to phytohemagglutinin and concanavalin A as a probe for T cell subset. *J. Immunol.* **110,** 362.

SERCARZ E.E. & BYERS V.S. (1967) The X-Y-Z scheme of immunocyte maturation. III. Early IgM memory and the nature of the memory cells. *J. Immunol.* **98,** 836.

TAMURA S., KURATA T., SUGIMOTO M. & EGASHIRA Y. (1973) Cellular and humoral immune responses in mice. I. Development of delayed-type footpad swelling against sheep erythrocytes and its suppression by intraperitoneal administration of the antigen. *Jap. J. med. Sci. Biol.* **26,** 161.

TAMURA S. & EGASHIRA Y. (1975) Cellular and humoral immune responses in mice. II. Effect of intraperitoneal or subcutaneous injection of carrier on anti-hapten antibody and delayed hypersensitivity responses. *Immunology,* **28,** 909.