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Initiation of Antibody Responses by Different Classes of Lymphocytes

IV. LYMPHOCYTES INVOLVED IN THE PRIMARY ANTIBODY RESPONSE TO A HAPTEN–PROTEIN CONJUGATE

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Summary. Thoracic duct cells and spleen cells from normal Lewis rats were tested for their ability to restore the antibody response of X-irradiated hosts to diphtheria toxoid (DTAP) or DNP-diphtheria toxoid (DNP–DT) adsorbed to alum. Although spleen cells restore a measurable response to both DTAP and DNP–DT, thoracic duct cells do not. However, thoracic duct cells from normal donors combined with small numbers of thoracic duct cells from donors primed to DTAP restore the primary anti-DNP response to DNP–DT. These findings indicate that lymphocytes which initiate the antibody response to the carrier play an important role in the initiation of the primary response to the hapten coupled to that carrier.

Further experiments show that the primary response of neonatally thymectomized rats to both DTAP and DNP–DT is markedly reduced as compared to that of age-matched normal rats. Thoracic duct cells can specifically restore the response of the thymectomized rats to DTAP, and, therefore, contain cells which can recognize and interact with the determinants of diphtheria toxoid. A combination of the latter cells and cells which can initiate the primary response to DNP is found in the thoracic duct lymph of normal rats. Yet, 'normal' thoracic duct cells cannot restore the response to DNP–DT in irradiated hosts. These results suggest that the complete immunologically competent unit for the carrier is required for the initiation of the primary response to a hapten–protein conjugate.

INTRODUCTION

Several investigators have shown that the determinants of the hapten and the carrier cooperate in the initiation of the primary antibody response to hapten–protein conjugates. Benacerraf, Green and Paul (1967) reported that DNP–polylysine does not elicit a primary response to the hapten in certain strains of guinea-pigs, but that DNP–polylysine complexed to bovine serum albumin does elicit a response to both the hapten and protein carrier. In similar studies, Rajewsky and Rottlander (1967) showed that the B_4 isoenzyme of porcine lactic dehydrogenase does not elicit a primary response in rabbits, but that the A_2B_2 hybrid isoenzyme elicits a response to both the A and B molecules. Schierman and McBride (1967) reported that different isoantigens of chicken erythrocytes also cooperate in the initiation of the primary antibody response in allogeneic hosts.

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Rajewsky, Schirrmacher, Nase and Jerne (1969) and Mitchison (1969) postulated a cellular basis for cooperation between the determinants of the hapten and carrier in the primary and secondary antibody responses to hapten–protein conjugates. The demonstration of synergy between cells primed to the hapten and cells primed to the carrier in the adoptive secondary response of mice supports this hypothesis (Mitchison, 1969). The latter finding suggests that the cells which interact with the carrier play an important role in the initiation of the adoptive antibody response to the hapten.

The present investigation was designed to study the requirements for cells which interact with the hapten and the carrier in the initiation of the primary response of rats to DNP-diphtheria toxoid (DNP–DT). In particular, we tested the ability of various lymphoid cells to restore the primary response of irradiated hosts to DNP–DT and to the free protein carrier (DT). The experimental findings suggest that the cells involved in the primary response to the carrier are required for the initiation of the primary response to the hapten.

MATERIALS AND METHODS

Animals

Inbred male Lewis rats (Microbiological Associates, Inc., Walkersville, Md) weighing 150–200 g were used throughout.

X-irradiation

Rats were placed in lucite containers and given 500 rad, whole body X-irradiation (15 mA; 200 kV; 54 cm source axis distance; 0·25 mm Cu + 0·55 Al filtration; dose rate 139 rad/min).

Collection of thoracic duct cells and spleen cells

The thoracic duct of 175–200 g rats was cannulated by a modification of the technique of Bollman, Cain and Grindlay (1948). Lymph was collected at 4° in 5 ml Krebs-Ringer solution containing 100 units heparin and 1 mg streptomycin. Details of the harvesting and injection of the cells have been described elsewhere (Strober, 1969). All collections of thoracic duct cells were made within 24 hours after cannulation of the thoracic duct.

Spleen cell suspensions were made in medium 199 according to the method of Billingham (1961).

Neonatal thymectomy

Thymectomy was performed within 24 hours of birth by a modification of the technique of Miller (1960). Rats which showed clinical signs of runting and those in which thymus remnants were found on routine post-mortem microscopic examination of the mediastinum were excluded from the study.

Induction of tolerance to diphtheria toxoid

Rats were made tolerant to diphtheria toxoid by the intraperitoneal injection of 50 Lf fluid diphtheria toxoid (kindly supplied by the Commonwealth of Massachusetts Department of Health) three times per week for 8–10 weeks, beginning from the date of birth. Animals were considered tolerant if they made no detectable antibody response to aluminum phosphate adsorbed diphtheria toxoid (DTAP) within 21 days after the injection of antigen (details of immunization are given below). Since putatively tolerant

rats were used as thoracic duct cell donors, DTAP was injected after 24 hours of thoracic duct drainage. Previous studies show that this period of drainage does not affect the primary response to DTAP (Strober, 1970 unpublished observations). Putatively tolerant thoracic duct cell donors which did produce a measurable antibody response were discarded from the study.

Coupling of DNP to diphtheria toxoid

DNP was coupled to diphtheria toxoid by reacting 2,4-dinitrobenzene sulphonate (Technical Grade, Eastman Organic Chemicals Co., Rochester, N.Y.) with fluid diphtheria toxoid for 8 hours at room temperature. The reaction was stopped by dialysis of the reaction mixture against several changes of distilled water. Spectrophotometric determinations (Eisen, Carsten and Belman, 1954) showed that eleven DNP groups were bound per molecule of protein. Adsorption of DNP-DT to precipitated alum was performed according to the method of Williams and Chase (1967). Approximately 10 mg of alum were used to adsorb 1 mg of protein.

Immunization procedures

Rats were immunized to DTAP (Parke, Davis & Co., Detroit, Mich.) or to DNP-DT adsorbed to alum by a single subcutaneous (0·25 ml) and intraperitoneal (0·25 ml) injection of 15 Lf (total) toxoid, or 0·4 or 0·1 mg (total) conjugate. Diphtheria toxoid preparations contained approximately 5 μg protein per Lf.

Antibody titrations

Titrations of antibodies to DTAP were performed in microtitre agglutination plates (Cooke Engineering Co., Alexandria, Va) by a previously described modification (Strober and Law, 1969) of the tanned red cell haemagglutination technique of Stavitsky (1954). Antibodies to DNP were determined by a modified Farr (1958) technique. Five-fold serial dilutions of antiserum were made in 10 × 75 mm test tubes in 0·4 ml of 10 per cent normal rat serum. Thereafter, 0·1 ml of 1×10^{-8}M [^{3}H] DNP-ethylamino caproic acid* ([^{3}H]DNP-EACA) was added to each tube. [^{3}H]DNP (New England Nuclear Corp., Boston, Mass.) was coupled to EACA by the technique of Green, Paul and Benacerraf (1966). The reaction mixture was allowed to stand for at least 1 hour at room temperature. A precipitate was formed by the addition of 0·5 ml saturated ammonium sulphate solution to each tube. The precipitate was spun down after 1–2 hours and 0·1 ml of the supernatant was counted in a liquid scintillation spectrometer. Antibody titres were taken as that dilution of antiserum which bound 33 per cent of the labelled antigen.

RESULTS

RESTORATION OF THE PRIMARY ANTIBODY RESPONSE OF X-IRRADIATED RATS TO DTAP AND TO DNP–DT

Thoracic duct cells and spleen cells from normal Lewis rats were tested for their ability to restore the antibody response of irradiated hosts to DTAP or DNP–DT. Recipients received 500 rad, whole body X-irradiation and a single intravenous injection of $2{\cdot}5 \times 10^8$ thoracic duct cells or 2×10^8 spleen cells 2 hours later. DTAP or DNP–DT were injected 24 hours after irradiation.

* Kindly supplied by Dr R. Asofsky, National Institute of Allergy and Infectious Diseases, Bethesda, Md 20014.

Fig. 1 shows that 500 rad abolished the primary response to the conjugate and to the free carrier. Although 2×10^8 spleen cells restored a measurable response to both the conjugate and carrier, $2{\cdot}5 \times 10^8$ thoracic duct cells did not (Fig. 1).

COOPERATION BETWEEN NORMAL THORACIC DUCT CELLS AND DIPHTHERIA TOXOID PRIMED THORACIC DUCT CELLS IN THE ADOPTIVE PRIMARY RESPONSE TO DNP–DT

Several experiments were performed to determine if thoracic duct cells primed to diphtheria toxoid can cooperate with normal thoracic duct cells in the restoration of the primary response of irradiated hosts to DNP–DT. Fig. 2 shows that 50×10^6 thoracic duct

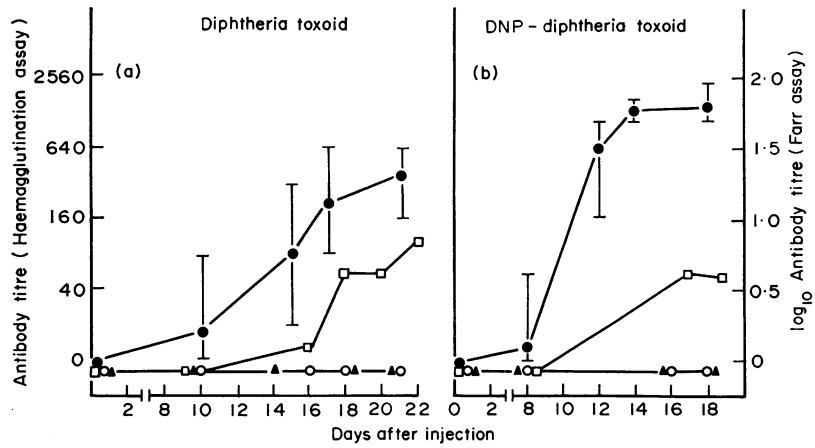

FIG. 1. Adoptive antibody response of rats to a single subcutaneous and intraperitoneal injection of 15 Lf DTAP or 0·4 mg DNP-DT. (a) ●, Mean tanned red cell haemagglutinin response of eight normal rats to DTAP with brackets showing range of titres; ▲, mean response of seven rats given 500 rad, whole body X-irradiation, 24 hours before injection of antigen; ○, mean response of eight rats given an intravenous injection of $2{\cdot}5 \times 10^8$ thoracic duct cells 2 hours after irradiation; DTAP was injected 24 hours after irradiation; □, mean response of seven rats given an intravenous injection of 1×10^8 spleen cells. (b) Mean $\log_{10}$ antibody titre which bound 33 per cent of [^{3}H]DNP-EACA in groups of six rats. ●, Mean response of normal rats to DNP–DT with brackets showing range of titres; ▲, mean response of rats given 500 rad whole body X-irradiation; ○, mean response of rats given $2{\cdot}5 \times 10^8$ thoracic duct cells 2 hours after irradiation; □, mean response of rats given 2×10^8 spleen cells.

cells from donors immunized to DTAP 3–4 months prior to cannulation of the thoracic duct restored a vigorous antibody response to DTAP and a minimal response to DNP–DT in irradiated hosts. The response restored by a combination of 50×10^6 thoracic duct cells primed to diphtheria toxoid and 2×10^8 normal thoracic duct cells approached the response of normal rats at day 16 and was considerably greater than the sum of the responses restored by either cell type alone (Fig. 2).

PRIMARY ANTIBODY RESPONSE OF NEONATALLY THYMECTOMIZED RATS TO DTAP AND TO DNP–DT

Normal and neonatally thymectomized Lewis rats were injected with DTAP and DNP–DT at 10–12 weeks of age. Fig. 3 shows that the primary antibody response of thymectomized rats is markedly reduced to both the conjugate and the free carrier as compared with

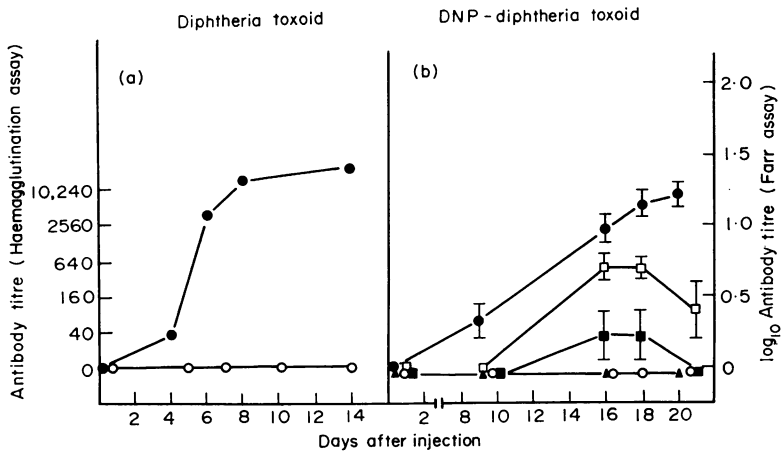

FIG. 2. Adoptive antibody response of rats to a single subcutaneous and intraperitoneal injection of 15 Lf DTAP or 0·1 mg DNP–DT. (a) ●, Mean tanned red cell haemagglutinin response of eight rats given 500 rad, whole body X-irradiation, and 50×10^6 DTAP primed thoracic duct cells 2 hours later; DTAP was injected 24 hours after irradiation; ○, mean response of seven rats given 500 rad and 50×10^6 DTAP primed thoracic duct cells; DTAP was not injected. (b) Mean $\log_{10}$ antibody titre which bound 33 per cent [^{3}H]DNP-EACA in groups of six rats; brackets show the standard error of the mean. ●, Mean response of normal rats to DNP-DT; ▲, mean response of rats given 500 rad, whole body X-irradiation; ○, mean response of rats given 2×10^8 'normal' thoracic duct cells 2 hours after irradiation; antigen was injected 24 hours after irradiation; ■, mean response of rats given 50×10^6 DTAP primed thoracic duct cells; □, mean response of rats given a combination of 2×10^8 'normal' thoracic duct cells and 50×10^6 DTAP primed thoracic duct cells.

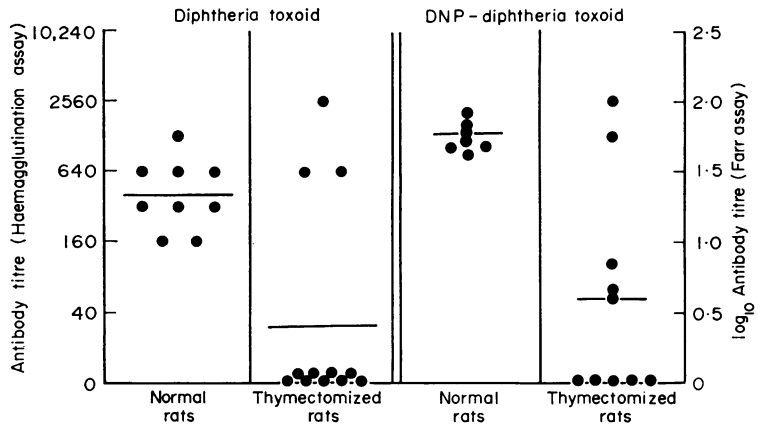

FIG. 3. Antibody response of normal or neonatally thymectomized rats 18 days after a single subcutaneous and intraperitoneal injection of 15 Lf DTAP or 0·4 mg DNP–DT. Horizontal line represents the mean antibody titre.

that of age-controlled normal rats. At least half of the thymectomized animals in each group showed no detectable antibody titre 18 days after the injection of antigen.

RESTORATION OF THE PRIMARY ANTIBODY RESPONSE OF NEONATALLY THYMECTOMIZED RATS TO DTAP

Thoracic duct cells from normal rats and rats rendered tolerant to diphtheria toxoid were tested for their ability to restore the primary antibody response of neonatally

thymectomized rats to DTAP. Donor cells were injected intravenously 22 hours prior to the challenge with DTAP. Fig. 4 shows that 2×10^8 thoracic duct cells from normal donors restored the primary response of thymectomized hosts, but an equal number of thoracic duct cells from tolerant donors did not.

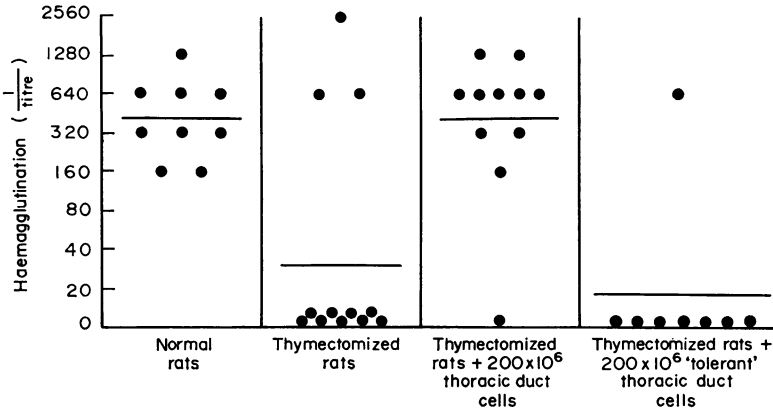

FIG. 4. Tanned red cell haemagglutinin response 21 days after a single subcutaneous and intraperitoneal injection of 15 Lf DTAP. Thoracic duct cells were given intravenously 22 hours before the injection of antigen. Horizontal line represents the mean antibody titre.

DISCUSSION

ARE THORACIC DUCT LYMPHOCYTES FROM NORMAL RATS ABLE TO RESTORE THE PRIMARY ANTIBODY RESPONSE OF IRRADIATED HOSTS TO DNP–DIPHTHERIA TOXOID (DNP–DT)?

Previous studies show that thoracic duct lymphocytes from normal rats are able to restore the primary antibody response of irradiated hosts to some antigens such as *S. typhi* flagella (Strober, 1969), but are unable to restore the response to other antigens such as aluminum phosphate adsorbed diphtheria toxoid (DTAP) (Strober and Law, 1969). The latter finding indicates that lymphocytes which are relatively fixed in the solid lymphoid tissues play an essential role in the initiation of the primary antibody response to DTAP.

In the present studies, thoracic duct cells and spleen cells were tested for their ability to restore the primary response of irradiated hosts to the hapten, DNP, coupled to diphtheria toxoid. The experimental results show that 2×10^8 spleen cells from normal donors are able to restore a measureable anti-DNP response, but $2{\cdot}5 \times 10^8$ thoracic duct cells are not. This suggests that the relatively fixed lymphocytes required for the restoration of the adoptive response to the diphtheria toxoid carrier are also required for the restoration of the response to DNP–DT.

ARE NORMAL THORACIC DUCT CELLS ABLE TO COOPERATE WITH DIPHTHERIA TOXOID PRIMED THORACIC DUCT CELLS IN THE ADOPTIVE RESPONSE TO DNP–DT?

The present study shows that $2{\cdot}5 \times 10^8$ thoracic duct cells from normal donors or 50×10^6 thoracic duct cells from donors immunized to DTAP restore a negligible anti-DNP response to DNP–DT. However, a combination of 2×10^8 normal cells and 50×10^6 diphtheria toxoid primed cells restores a vigorous response which is considerably greater

than the sum of the responses restored by either cell type independently. These findings demonstrate that normal thoracic duct cells can cooperate with carrier primed cells in the initiation of the adoptive primary response to DNP–DT. In addition, the findings suggest that normal thoracic duct cells are unable to restore the primary response to the conjugate because they lack cells which are capable of initiating the adoptive antibody response to the carrier.

ARE THE EFFECTS OF NEONATAL THYMECTOMY ON THE PRIMARY RESPONSE OF RATS TO DTAP AND TO DNP–DT SIMILAR?

Neonatally thymectomized or normal rats were injected with DTAP or DNP–DT at 10–12 weeks of age. The primary response of thymectomized rats to both DTAP and DNP–DT was markedly reduced as compared to that of the age-controlled normal rats. These findings indicate that 'thymus derived' cells (Mitchell and Miller, 1968) are required for the initiation of the primary response to the free carrier and to the hapten–protein conjugate.

ARE THORACIC DUCT CELLS FROM NORMAL RATS ABLE TO SPECIFICALLY RESTORE THE ANTIBODY RESPONSE OF THYMECTOMIZED RATS TO DTAP?

Our previous studies show that thoracic duct cells from normal rats are able to restore the primary antibody response of neonatally thymectomized hosts to DTAP (Strober and Law, 1969). However, control experiments testing the immunological specificity of the restoration were not performed. The present study compares the ability of thoracic duct cells from normal and tolerant donors to restore the primary response of thymectomized hosts to DTAP. Although 'normal' thoracic duct cells restore the response, 'tolerant' cells have no restorative action. These findings show that normal thoracic duct lymphocytes can recognize and interact with the antigenic determinants of diphtheria toxoid.

ARE THE CELLS REQUIRED FOR THE INITIATION OF THE PRIMARY ANTIBODY RESPONSE TO THE CARRIER (DIPHTHERIA TOXOID) ALSO REQUIRED FOR THE INITIATION OF THE PRIMARY RESPONSE TO THE HAPTEN (DNP)?

The present studies show that rats lacking certain types of lymphocytes are unable to make a primary antibody response to diphtheria toxoid or to DNP coupled to diphtheria toxoid. In addition, these studies show that the inability of normal thoracic duct cells to restore the primary adoptive response of irradiated rats to DNP–DT can be corrected by the addition of cells which can restore the response of irradiated hosts to the free carrier (DT). These findings indicate that the cells which initiate the primary response to the carrier are required for the initiation of the primary response to the hapten coupled to that carrier. These results differ significantly from those noted in studies of the adoptive secondary antibody response to the DNP hapten, since hapten primed cells are able to restore a vigorous secondary anti-DNP response to alum adsorbed DNP-DT in the absence of carrier primed cells (Strober, 1970).

Although normal thoracic duct lymphocytes are unable to restore the primary response of irradiated hosts to DTAP, they are able to specifically restore the primary response of thymectomized hosts. This finding shows that there are considerable numbers of 'thymus

derived' cells in the thoracic duct lymph which can recognize and interact with the determinants of diphtheria toxoid. A combination of the latter cells and cells which can initiate the response to DNP is found in the thoracic duct lymph of normal rats, yet normal thoracic duct cells cannot restore the adoptive response to DNP–DT in irradiated hosts. This suggests that the cells which interact with the hapten must cooperate with the complete immunologically competent unit* for the carrier in order to restore the primary response to the conjugate. The competent unit for diphtheria toxoid may include circulating 'thymus derived' cells and relatively fixed 'bone marrow derived' cells (Strober and Law, 1969). The restorative activity of the spleen may be related to the presence of these two cell types in the solid lymphoid tissues. Quantitative considerations, however, may also explain the inactivity of thoracic duct cells. The ratio of cells which recognize the carrier and cells which initiate the response to the hapten may be sub-optimal in the thoracic duct lymph, but may be optimal in the spleen.

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* An immunologically competent unit can be defined as a single cell or minimum combination of different cells which can restore the ability of an immunologically inert host to make an immune response to a given immunogen (Strober and Law, 1969).