INITIATION OF ANTIBODY RESPONSES BY DIFFERENT CLASSES OF LYMPHOCYTES

I. Types of Thoracic Duct Lymphocytes Involved in Primary Antibody Responses of Rats

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(Received for publication 2 June 1969)

Early studies by Gowans and his colleagues show that circulating small lymphocytes can restore the primary hemolysin response of X-irradiated rats to sheep red blood cells (1, 2). In addition, their studies with lymphocytes from tolerant donors show that restoration depends upon the interaction between donor cells and antigen (1, 3). These studies strongly suggest that circulating small lymphocytes are the precursors of hemolysin-forming cells in the rat.

Similar cell transfer studies by Nossal et al. (4, 5) show that circulating lymphocytes from mice carrying the T6 chromosome marker differentiate into hemolysin-forming cells in X-irradiated hosts challenged with sheep red blood cells. This is a direct demonstration that circulating lymphocytes not only restore the primary hemolysin response of X-irradiated hosts but also produce the hemolysin-forming cells.

We attempted to extend the conclusions drawn from the experiments of Gowans and Nossal by carrying out several cell transfer studies with purified protein antigens as well as with sheep red blood cells. Studies with tetanus and diphtheria toxoids unexpectedly show that circulating lymphocytes are unable to restore the primary antibody response of X-irradiated rats to these antigens (6–8). These experiments indicate that noncirculating lymphocytes initiate the primary antibody response to the toxoids.

Preliminary experiments with bovine serum albumin, horse spleen ferritin, and *Salmonella typhi* flagella show that thoracic duct cells are able to restore the primary response to these antigens. However, no conclusion can be drawn as to the classes of lymphocytes which initiate these responses, since 5–10% of thoracic duct cells are dividing, short-lived, medium to large lymphocytes which do not recirculate (9, 10). The object of the present study is to determine whether the medium and large lymphocytes play a part in the initiation of primary antibody responses to these three protein antigens.

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Materials and Methods

Animals.—Inbred male Lewis rats (Microbiological Associates, Inc., Bethesda, Md.) weighing 150–200 g were used in all experiments.

X-irradiation.—Two Westinghouse Quadrocondex units (15 ma; 200 kv; 54 cm Source Axis Distance (SAD); 0.25 mm Cu + 0.55 Al filtration; dose rate 139 rpm) were used to administer 500 R, whole body X-irradiation, to rats placed in cylindrical lucite containers.

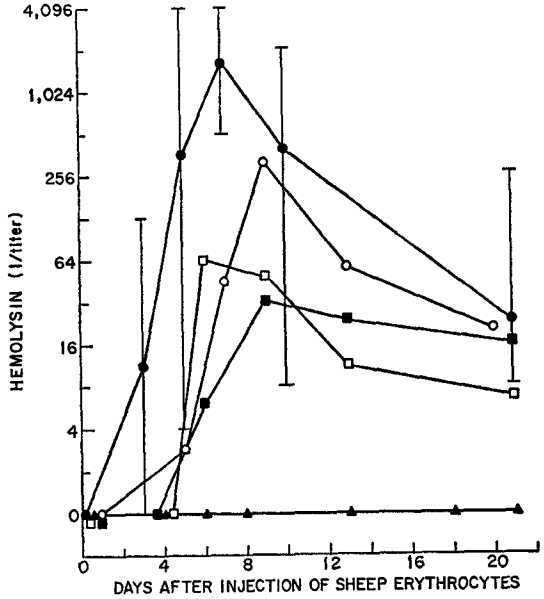

FIG. 1. Hemolysin response to a single intravenous injection of 10^8 sheep red blood cells (SRBC). ●——●, mean response of seven normal rats, with brackets showing range of titers; ▲——▲, mean response of eight rats given 500 R, whole body X-irradiation, 24 hr before injection of SRBC; ○——○, mean response of eight rats given an intravenous injection of 2.5×10^8 thoracic duct cells 2 hr after irradiation, SRBC were injected 24 hr after irradiation; □——□, mean response of six rats given an intravenous injection of 10^8 thoracic duct cells; ■——■, mean response of six rats given an intravenous injection of 10^8 spleen cells.

Collection of Thoracic Duct Cells and Spleen Cells.—The thoracic duct of 175–200 g normal (unimmunized) rats was cannulated by a modification of the technique of Bollman, et al. (11). After the operation, rats were maintained unanesthetized in restraining cages. A Krebs-Ringer solution containing streptomycin (0.1 mg/ml) and heparin (1 unit/ml) was infused intravenously at 2 ml/hr during periods of lymph collection. Thoracic duct lymph was collected in sterile flasks containing 5 ml Krebs-Ringer solution with 100 units heparin and 1 mg streptomycin. The flasks were kept at room temperature for 12-hr collections, or in an ice bath for 24-hr collections. Thoracic duct cells were harvested from the lymph by centrifugation at 150 g for 10 min and resuspended in tissue culture medium 199 (Microbiological Associates, Inc., Bethesda, Md.). All collections of thoracic duct cells were made within 24 hr after cannulation of the thoracic duct.

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Spleen cell suspensions were made in medium 199 according to the method of Billingham (12). Both thoracic duct cell and spleen cell suspensions were injected into the lateral tail vein of prospective recipients.

In Vitro Incubation of Thoracic Duct Cells.—In several experiments, thoracic duct cells were incubated in vitro for 24 hr at 37°C according to the technique of Gowans and Uhr (13). This procedure preferentially destroys the large dividing lymphocytes present in thoracic duct lymph (13). Thoracic duct cells were harvested from 12-hr collections of lymph and washed

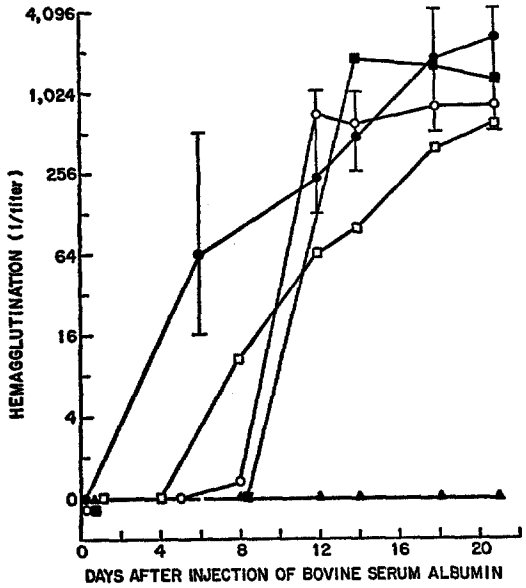

FIG. 2. Tanned red cell hemagglutinin response to a single subcutaneous injection of 1 mg of BSA in complete Freund's adjuvant. ●——●, mean response of seven normal rats, with brackets showing range of titers; ▲——▲, mean response of seven rats given 500 R, whole body X-irradiation, 24 hr before the injection of BSA; ○——○, mean response of six rats given an intravenous injection of 10^8 thoracic duct cells 2 hr after irradiation, BSA was injected 24 hr after irradiation; □——□, mean response of six rats given an intravenous injection of 0.5×10^8 thoracic duct cells; ■——■, mean response of six rats given an intravenous injection of 10^8 spleen cells.

once in Krebs-Ringer solution. The cells were subsequently suspended in a medium containing 79% (v/v) medium 199, 20% phosphate-buffered saline, pH 7.3, and 1% inactivated normal rat serum. The concentration of cells was adjusted to $5–10 \times 10^7$ cells per ml, and the suspension was gently agitated in a conical flask sealed with a rubber stopper. At the end of the incubation period the cells were harvested by centrifugation at 150 g for 10 min and resuspended in medium 199 for intravenous injection.

Approximately 50–85% of thoracic duct cells were recovered after incubation. We arbitrarily discarded experiments in which the cell survival was less than 90%, judging by trypan blue exclusion, and those in which cell recovery was less than 75%. Six differential counts of thoracic duct cells prior to incubation showed 34–79 large lymphocytes present per 1,000 cell count. An equal number of counts of incubated cells showed 0–5 large lymphocytes present per 1,000 cell count.

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Vinblastine Treatment of Thoracic Duct Cell Donors.—A single intraperitoneal injection (0.3 mg in 1 ml saline) of the mitotic inhibitor vinblastine sulfate (Eli Lilly and Co., Indianapolis, Ind.) was administered to several rats 24 hr prior to cannulation of the thoracic duct. Mild diarrhea was occasionally noted prior to the operation, but all animals survived the procedure without difficulty. However, the output of lymph and cells from the thoracic duct was frequently diminished. Thoracic duct cells used in the present studies were obtained from donors with either low or normal outputs of cells. Six differential counts of thoracic duct cells

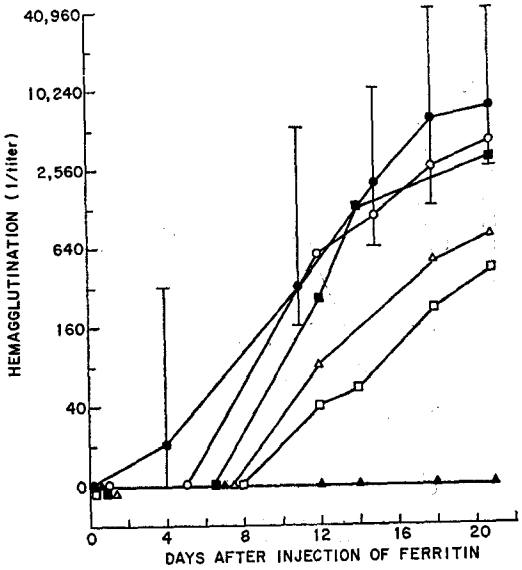

FIG. 3. Tanned red cell hemagglutinin response to a single subcutaneous injection of 2 mg of HSF in complete Freund's adjuvant. ●——●, mean response of eight normal rats, with brackets showing range of titers; ▲——▲, mean response of six rats given 500 R, whole body X-irradiation, 24 hr before the injection of HSF; ○——○, mean response of six rats given an intravenous injection of 2.5×10^8 thoracic duct cells 2 hr after irradiation; HSF was injected 24 hr after irradiation. △——△, mean response of six rats given an intravenous injection of 10^8 thoracic duct cells; □——□, mean response of five rats given an intravenous injection of 0.5×10^8 thoracic duct cells; ■——■, mean response of six rats given an intravenous injection of 10^8 spleen cells.

issuing from these donors showed 0–12 large lymphocytes present per 1,000 cell count. Collections of lymph were made for 24 hr, and cells were prepared for intravenous injection as described for normal donors.

Counting of Lymphoid Cells.—Thoracic duct cells and spleen cells were counted in a standard Neubauer hemocytometer. Differential counts of thoracic duct cells were performed on living cells placed in the hemocytometer and on Giemsa-stained smears. Thoracic duct cells greater than 8 μ in diameter were classified as large lymphocytes.

Immunization Procedures.—Rats were immunized to sheep red blood cells (SRBC)[1] by a

[1] Abbreviations used in this article: SRBC, sheep red blood cells; BSA, bovine serum albumin; HSF, horse spleen ferritin.

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single intravenous injection of 10^8 cells in 1 ml saline. Sheep cells were collected in Alsever's solution and washed three times in saline prior to injection. Immunization to bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio) or horse spleen ferritin (cadmium free, Pentex Inc., Kankakee, Ill.) in complete Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.) was accomplished by the injection of 0.1 ml of emulsion into each hind footpad to give a total dose of 1 mg BSA or 2 mg HSF. Equal volumes of antigen in saline and complete Freund's adjuvant were used to prepare the emulsion. Immunization to *Salmonella typhi* flagella, strain TY2W,[2] was achieved by a single intravenous injection of 5 μg flagella in 1 ml saline. The flagella were prepared by a modification of the method of Kobayashi et al. (14).

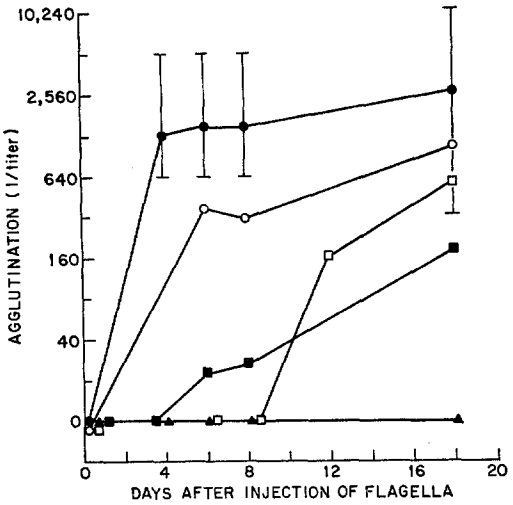

FIG. 4. Agglutinin response to a single intravenous injection of 5 μg *Salmonella typhi* flagella. ●——●, mean response of eight normal rats with brackets showing range of titers; ▲——▲, mean response of seven rats given 500 R, whole body X-irradiation, 24 hr before the injection of flagella; ○——○, mean response of six rats given an intravenous injection of 2.5×10^8 thoracic duct cells 2 hr after irradiation; flagella were injected 24 hr after irradiation. □——□, mean response of six rats given an intravenous injection of 0.5×10^8 thoracic duct cells; ■——■, mean response of eight rats given an intravenous injection of 0.5×10^8 spleen cells.

Antibody Titrations.—Sheep erythrocyte hemolysin titrations were performed in microtiter agglutination plates (Cooke Engineering Co., Alexandria, Va.) by the method of McGregor and Gowans (15). Antibodies to BSA and HSF were also measured in microtiter plates using the tanned red cell hemagglutination technique of Stavitsky (16). Titration of agglutinins to *Salmonella typhi* flagella was performed in 10×75 mm test tubes as described by Campbell et al. (17). After incubation of the whole organisms with diluted antisera for one-half hr at room temperature, the suspensions were centrifuged at 500 g for 10 min. Test tubes were subsequently agitated and end points were read as the highest dilution of antisera showing macroscopic clumping.

[2] Kindly supplied by Dr. R. Hunter, National Institute for Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

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Collection of Serum for Antibody Titrations.—Blood was collected by incision of the tail. Samples remained at room temperature for 30 min before refrigeration for 24 hr. Serum was separated from the clot by centrifugation at 300 g for 15 min and stored at −20°C.

RESULTS

Restoration of Primary Antibody Responses by Spleen Cells and Thoracic Duct Cells.—Lewis rats received 500 R, whole body X-irradiation, and a single intravenous injection of thoracic duct cells or spleen cells 2 hr later. Antigen

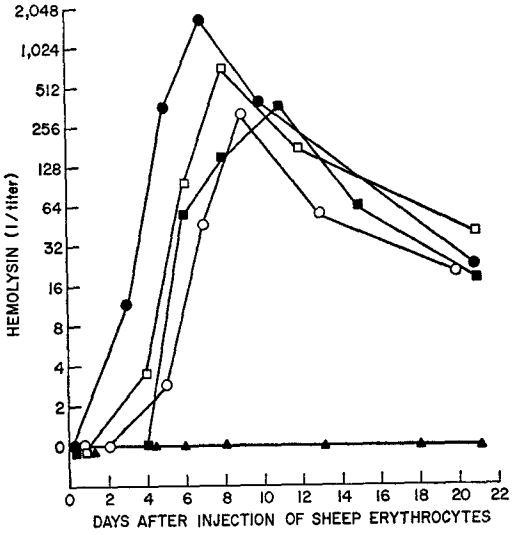

FIG. 5. Hemolysin response of rats given an intravenous injection of 2.5×10^8 thoracic duct cells and 10^8 sheep red blood cells 2 hr and 24 hr respectively after irradiation. ○——○, mean response of eight rats given normal thoracic duct cells; ■——■, mean response of six rats given incubated thoracic duct cells; □——□, mean response of six rats given thoracic duct cells from donors treated with vinblastine; ▲——▲, mean response of eight rats given no thoracic duct cells is shown for comparison. ●——●, mean response of seven normal rats is shown for comparison.

was injected 24 hr after irradiation. The primary antibody response restored by 50, 100, and 250×10^6 thoracic duct cells was compared to that restored by 50 or 100×10^6 spleen cells.

Fig. 1 shows that 500 R abolished the primary hemolysin response to SRBC. The peak titers restored by equal numbers of thoracic duct cells or spleen cells were similar. Thoracic duct cells produced a somewhat earlier rise in the hemolysin titers.

The kinetics of the antibody response to BSA were similar when 100×10^6 spleen cells or 100×10^6 thoracic duct cells were used (Fig. 2). The peak titer was slightly higher with the spleen cells. The response restored by 100×10^6

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thoracic duct cells was identical with that restored by 250 × 10^6 cells. However, 50 × 10^6 cells produced a slowly rising response which approached the titers of the two higher doses of cells at 21 days (Fig. 2).

Fig. 3 shows that 100 × 10^6 spleen cells were as efficient as 250 × 10^6 thoracic duct cells in restoring the antibody response to HSF. The response produced by 50 × 10^6 thoracic duct cells rose slowly and remained well below that produced by 250 × 10^6 thoracic duct cells at 21 days (Fig. 3).

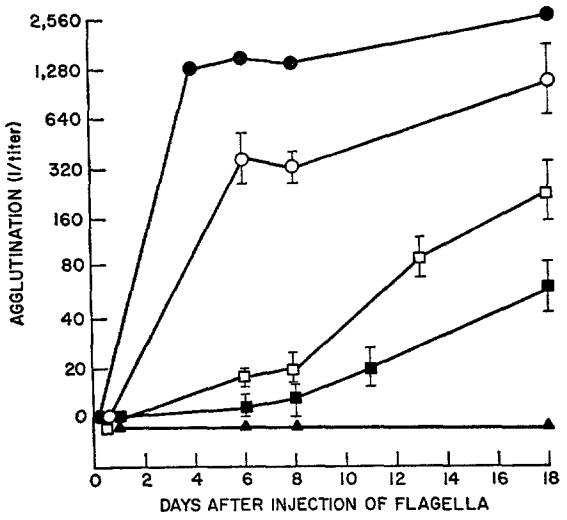

FIG. 6. Agglutinin response of rats given an intravenous injection of 2.5 × 10^8 thoracic duct cells and 5 μg of *Salmonella typhi* flagella 2 hr and 24 hr respectively after irradiation. O——O, mean response of six rats given normal thoracic duct cells; ■——■, mean response of six rats given incubated thoracic duct cells; □——□, mean response of six rats given thoracic duct cells from donors treated with vinblastine; ▲——▲, mean response of seven rats given no thoracic duct cells is shown for comparison. ●——●, mean response of eight normal rats is shown for comparison. Brackets show the standard error of the mean.

The *Salmonella typhi* agglutinin response restored by 50 × 10^6 spleen cells or 50 × 10^6 thoracic duct cells was barely detectable during the 1st wk (Fig. 4). However, the response rose rapidly during the 2nd wk and began to approach that restored by 250 × 10^6 thoracic duct cells at 18 days (Fig. 4).

Restoration of Primary Antibody Responses by Thoracic Duct Cells Lacking Large Dividing Lymphocytes.—In several experiments thoracic duct cells lacking large dividing lymphocytes were tested for their ability to restore the primary antibody responses of X-irradiated hosts. Two procedures were used to reduce the number of large lymphocytes present in thoracic duct cell inocula. In the first procedure, thoracic duct cells from normal donors were incubated in vitro for 24 hr at 37°C. In the second, thoracic duct cell donors were treated

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with the mitotic inhibitor vinblastine sulfate 24 hr prior to cannulation of the thoracic duct.

Fig. 5 shows that inocula lacking large dividing lymphocytes restored the primary hemolysin response to SRBC as well as normal thoracic duct cells. However, when *Salmonella typhi* flagella or HSF were used as antigen, the former cells were approximately 5 times less efficient in restoring the primary

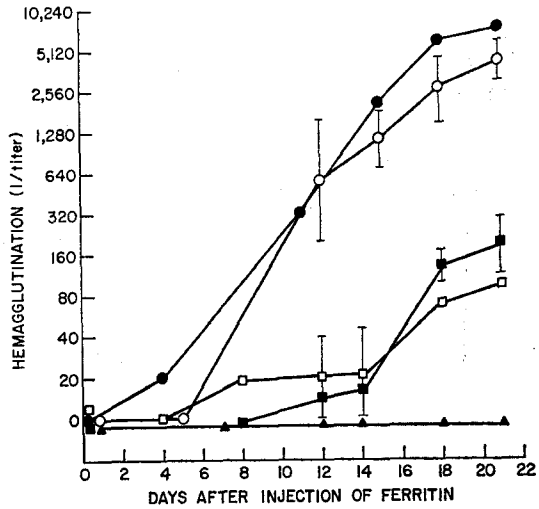

FIG. 7. Tanned red cell hemagglutinin response of rats given an intravenous injection of 2.5×10^8 thoracic duct cells and a subcutaneous injection of 2 mg of HSF in complete Freund's adjuvant 2 hr and 24 hr respectively after irradiation. O——O, mean response of six rats given normal thoracic duct cells; ■——■, mean response of six rats given incubated thoracic duct cells; □——□, mean response of eight rats given thoracic duct cells from donors treated with vinblastine; ▲——▲, mean response of six rats given no thoracic duct cells is shown for comparison. ●——●, mean response of eight normal rats is shown for comparison. Brackets show the standard error of the mean.

response than the latter (compare Figs. 2 and 3 with Figs. 6 and 7). Differences between the antibody titers restored by the different inocula were statistically significant ($P < 0.001$) as judged by the Student t test. Some recipients of thoracic duct cells lacking large lymphocytes showed no detectable antibody titers for 21 days. No attempt was made to correlate the degree of depression of the antibody response with the number of large lymphocytes received by individual animals.

Normal thoracic duct cells were more efficient than incubated cells in restoring the primary response to BSA (Fig. 8). However, the response produced by cells from vinblastine-treated donors rose more rapidly but reached a somewhat lower plateau than that produced by normal cells.

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DISCUSSION

Thoracic duct cells and spleen cells were tested for their ability to restore the primary antibody response of X-irradiated rats to BSA, SRBC, HSF, and *Salmonella typhi* flagella. Thoracic duct cells were able to form immunologically competent *units* for all the antigens studied. An immunologically competent *unit* can be defined as a single cell or as a minimum combination of different

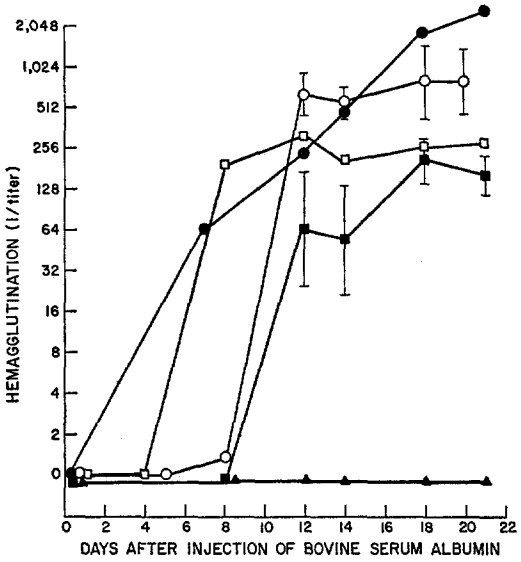

FIG. 8. Tanned red cell hemagglutinin response of rats given an intravenous injection of thoracic duct cells and a subcutaneous injection of 1 mg of bovine serum albumin in complete Freund's adjuvant 2 hr and 24 hr respectively after irradiation. O——O, mean response of six rats given 10^8 normal thoracic duct cells; ■——■, mean response of six rats given 2.5×10^8 incubated thoracic duct cells; □——□, mean response of eight rats given 2.5×10^8 thoracic duct cells from donors treated with vinblastine; ▲——▲, mean response of seven rats given no thoracic duct cells is shown for comparison. ●——●, mean response of seven normal rats is shown for comparison. Brackets show the standard error of the mean.

cells which can restore the ability of an immunologically inert host to produce an immune response to a given immunogen (8). This definition implies that the unit can recognize either the unaltered or processed immunogen and can subsequently differentiate into antibody-forming cells. The experimental findings suggest that for some antigens spleen cells may contain as many competent units as thoracic duct cells, since the former cells were at least as efficient as the latter in restoring the primary responses to BSA, HSF, and *Salmonella typhi* flagella.

A possible explanation of the relatively high efficiency of spleen cells is that the immunologically competent units may contain cells other than circulating small lymphocytes. To investigate this possibility, we tested thoracic duct cells

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lacking large dividing lymphocytes for their ability to restore the primary antibody responses of irradiated hosts. Large lymphocytes were eliminated by the in vitro incubation of thoracic duct cells for 24 hr at 37°C or by treatment of thoracic duct cell donors with the mitotic inhibitor vinblastine sulfate 24 hr prior to cannulation of the thoracic duct. Gowans and Uhr (13) have shown that the former procedure preferentially destroys large lymphocytes, presumably on the basis of heat sensitivity. Syeklocha et al. have shown that the latter procedure preferentially destroys rapidly dividing lymphoid cells (18). Sensitivity to the drug is related to the proliferative rate of the cells.

Experiments with SRBC show that incubated cells and cells from vinblastine treated donors are as efficient as normal thoracic duct cells in restoring the primary hemolysin response. These results suggest that for SRBC the immunologically competent unit is composed exclusively of nondividing small lymphocytes. Similar results with incubated cells in rats have already been reported by Gowans and his colleagues (1–3). In addition, Syeklocha et al. have shown that spleen cells from vinblastine-treated mice are as efficient as spleen cells from normal mice in restoring the primary hemolysin response (18).

On the other hand, experiments with HSF and *Salmonella typhi* flagella show that incubated cells and cells from vinblastine-treated donors are about five times less efficient than normal thoracic duct cells in restoring the primary antibody response. These results suggest that large dividing lymphocytes present in thoracic duct lymph play a part in the immunologically competent unit for HSF and *Salmonella typhi* flagella. Nossal et al. have shown that thoracic duct cells depleted of large lymphocytes by passage through a glass bead column are also less efficient than normal thoracic duct cells in restoring the agglutinin response of X-irradiated mice to *Salmonella adelaide* flagella (4). However, these results were interpreted with caution, since only 10% of the cells applied to the columns were recovered. In the present experiments, at least 75% of thoracic duct cells were recovered after the incubation procedure. Nevertheless the present findings do not rule out the possibility that the unit for flagella or HSF may be composed of small lymphocytes which are sensitive to both incubation and vinblastine treatment.

Incubated cells were considerably less efficient than normal thoracic duct cells in restoring the primary response to BSA. However, cells from vinblastine-treated donors were more efficient than normal thoracic duct cells in restoring the early response. These findings indicate that the immunologically competent unit for BSA is formed from classes of lymphocytes which are sensitive to heat but are not rapidly dividing, i.e., nondividing, heat-sensitive, small lymphocytes or slowly dividing, heat-sensitive, medium lymphocytes. These cells differ from those which form the units for SRBC, HSF, and *Salmonella typhi* flagella.

In conclusion, the experimental results show that different classes of lymphocytes are involved in the initiation of primary antibody responses to different

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antigens. The heterogeneity of these classes is probably extensive in view of the differences between the present findings and those obtained previously with tetanus and diphtheria toxoids (6–8). A possible explanation of these findings is that several classes of lymphocytes present in the rat can interact with each antigen studied. Differences in the processing of each antigen, however, may select out different classes of lymphocytes for subsequent interaction with the altered antigen. Evidence for the presence of more than one class of lymphocyte which can interact with a given antigen has already been reported in studies of the primary antibody response to *Salmonella adelaide* flagellin in the mouse (19) and of the secondary antibody response to SRBC in the rat (2).

SUMMARY

Thoracic duct cells and spleen cells were tested for their ability to restore the primary antibody response of X-irradiated rats to bovine serum albumin (BSA), sheep red blood cells (SRBC), horse spleen ferritin (HSF), and *Salmonella typhi* flagella. Spleen cells were at least as efficient as thoracic duct cells in restoring the response to BSA, HSF, and *Salmonella typhi* flagella.

In further experiments thoracic duct cells lacking large dividing lymphocytes were tested for their ability to restore the primary response. Large lymphocytes were eliminated by the in vitro incubation of thoracic duct cells for 24 hr at 37°C or by treatment of thoracic duct cell donors with the mitotic inhibitor vinblastine sulfate 24 hr prior to cannulation of the thoracic duct.

Experiments with SRBC show that incubated cells and cells from vinblastine-treated donors are as efficient as normal cells in restoring the primary antibody response. On the other hand, experiments with HSF and *Salmonella typhi* flagella show that incubated cells and cells from vinblastine-treated donors are about five times less efficient than normal cells in restoring the response. Normal thoracic duct cells were more efficient than incubated cells but less efficient than cells from vinblastine-treated donors in restoring the early response to BSA.

The experimental findings indicate that the classes of thoracic duct lymphocytes which initiate the primary antibody response to SRBC differ from the classes which initiate the response to HSF and *Salmonella typhi* flagella, or BSA.

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