FURTHER STUDIES ON THE ROLE OF CIRCULATING LYMPHOCYTES IN THE INITIATION OF PRIMARY ANTIBODY RESPONSES TO DIFFERENT ANTIGENS

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Abstract.—Thoracic duct cells obtained from normal (unimmunized) donors restored the primary hemolysin response of lethally irradiated or neonatally thymectomized rats to sheep red blood cells. Synergy between thoracic duct cells and bone-marrow cells was demonstrated in the irradiated hosts. However, thoracic duct cells did not restore the primary antibody response of irradiated rats challenged with diphtheria toxoid, but did restore the response of neonatally thymectomized rats. The addition of peritoneal exudate cells or bone-marrow cells to inocula of thoracic duct cells also failed to restore the response of irradiated hosts to diphtheria toxoid, although normal spleen cells restored the response. These findings indicate that the cellular events involved in the initiation of the primary antibody response to sheep red blood cells differ from those involved in the response to diphtheria toxoid.

Although circulating lymphocytes are able to restore the primary antibody response of *X*-irradiated rats to some antigens, they are unable to restore the response to others.[1, 2] Differences in the restorative activity are related to the functional abilities of the lymphocytes, and not to differential damage of macrophage function or antigen processing in the *X*-irradiated hosts.[1]

In order to investigate further the role of circulating lymphocytes in immune responses to different antigens, we tested the ability of thoracic duct cells to restore primary antibody responses of neonatally thymectomized rats. In addition, we tested the ability of these cells to synergize with other lymphoid cells in restoring the primary responses of *X*-irradiated rats.

Six antigens—bovine serum albumin, horse spleen ferritin, sheep red blood cells, tetanus toxoid, diphtheria toxoid, and *Salmonella typhosa* flagella—are presently under investigation in our laboratory. In preliminary experiments, only sheep red blood cells and diphtheria toxoid showed significantly depressed primary antibody responses in neonatally thymectomized Lewis rats.[3] We therefore used the latter antigens in comparative studies of the immunological functions of thoracic duct cells presented here.

Materials and Methods.—Inbred Lewis rats (Microbiological Associates, Inc., Walkersville, Md.) were used throughout. Thymectomy was performed within 24 hr of birth by a modification of the technique of Miller.[4] Rats which showed clinical signs of runting and those in which thymus remnants were found on routine post-mortem microscopic examination of the anterior mediastinum were excluded from the study. Two Westinghouse Quadrocondex units (15 ma; 200 kv, 54 cm, SAD; 0.25 mm Cu + 0.55 mm Al filtration; dose rate 139 rpm) were used for whole-body X irradiation. Thoracic duct cells were obtained with the technique of Bollman *et al.*[5] Spleen and bone-marrow cell suspensions were prepared by the method of Billingham.[6] Rats were made tolerant to sheep red blood cells by the injection of 2.5×10^9 cells (0.2 ml, 1×10^9 cells intravenously, and 03.

ml, 1.5 × 10^9 cells intraperitoneally) a few hours after birth and 2.5 × 10^9 cells intraperitoneally two times per week thereafter.

Rats were immunized to sheep erythrocytes by a single intravenous injection of 1 × 10^8 cells. Immunization to alum-precipitated diphtheria toxoid (Parke, Davis, & Co., Detroit) was achieved by a single intraperitoneal (0.5 ml, 7.5 Lf) and subcutaneous (0.5 ml, 7.5 Lf) injection of 15 Lf toxoid. Antibody titrations were performed in microtiter agglutination plates (Cooke Engineering Co., Alexandria, Va.). Sheep red blood cell hemolysins were titrated according to the method of McGregor and Gowans.[7] Antibody to diphtheria toxoid was measured by a modification of the tanned red cell hemagglutination technique of Stavitsky.[8] Red cells were tanned a few hours after collection and sensitized with fluid diphtheria toxoid (kindly supplied by the Commonwealth of Massachusetts Department of Health) for 10 min at room temperature at a concentration of 30 Lf/ml. Serum dilutions were made in 1% normal rabbit serum.

Results.—Restoration of the primary hemolysin response of X-irradiated rats with thoracic duct cells: The hemolysin response of normal adult male Lewis rats to sheep erythrocytes is shown in Figure 1. The response was abolished by exposure to 500 r whole-body X-irradiation 24 hours prior to the injection of the erythrocytes and was restored by the intravenous injection of 2.5 × 10^8 thoracic duct cells two hours after irradiation (Fig. 1). Thoracic duct cells were obtained from normal (unimmunized) adult male rats.

In order to determine whether restoration depended upon the presence of host bone-marrow cells which were not destroyed by sublethal irradiation, the experiments were repeated with a lethal dose of X irradiation (900 r). Figure 1 shows that increasing the dose of irradiation did not alter the ability of thoracic

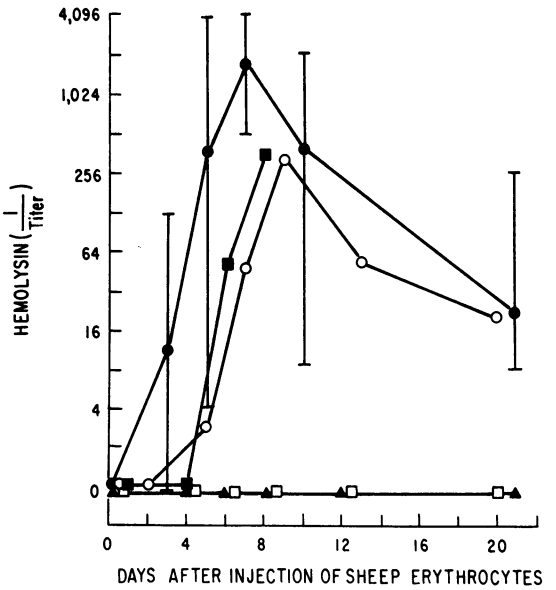

FIG. 1.—Hemolysin response to a single intravenous injection of 1 × 10^8 sheep red blood cells.

●—●, Mean response of seven normal rats; brackets show range of titers.

▲—▲, Mean response of six rats exposed to 500 r whole-body irradiation 24 hr before injection of sheep red blood cells.

○—○, Mean response of six rats given an intravenous injection of 2.5 × 10^8 thoracic duct cells 2 hr after irradiation (500 r); sheep red blood cells were injected 24 hr after irradiation.

□—□, Mean response of six rats given an intravenous injection of 2.5 × 10^8 "tolerant" thoracic duct cells (obtained from six rats made neonatally tolerant to sheep red blood cells) 2 hr after irradiation (500 r); sheep red blood cells were injected 24 hr after irradiation.

■—■, Mean response of six rats given an intravenous injection of 2.5 × 10^8 thoracic duct cells 2 hr after lethal irradiation (900 r); sheep red blood cells were injected 24 hr after irradiation.

duct cells to restore the response. Similar results have already been reported for rats by Ellis *et al.*[9]

Restoration of the primary hemolysin response of neonatally thymectomized rats: The depressed primary hemolysin response of neonatally thymectomized Lewis rats challenged with sheep erythrocytes at four weeks of age is shown in Figure 2. An inoculum of 2×10^8 thoracic duct cells injected intravenously 22 hours prior to the injection of sheep erythrocytes restored the response (Fig. 2). In order to determine whether restoration was due to a specific immunological function of the donor cells, thoracic duct cells obtained from adult rats made tolerant to sheep erythrocytes were used in several experiments. Tolerance was determined by the absence of serum hemolysins and by the inability of thoracic duct cells to restore a measurable primary response in sublethally irradiated hosts (Fig. 1). Figure 2 shows that "tolerant" thoracic duct cells did not restore the primary hemolysin response of neonatally thymectomized rats.

Synergism between thoracic duct cells and bone-marrow cells: Figure 3 shows the hemolysin response of sublethally irradiated rats reconstituted with 1×10^8 thoracic duct cells alone, 1×10^8 bone-marrow cells alone, and a combination of 1×10^8 thoracic duct cells and 1×10^8 bone-marrow cells. The mean total titer (mean of all titers measured on days 4, 6, 8, 13, and 21) produced by the combination of thoracic duct cells and bone-marrow cells was significantly greater ($p < 0.005$), according to the student's t test, than the sum of that produced by the two cell types independently. These results confirm the recent observations for mice made by Mitchell and Miller.[10]

Inability of thoracic duct cells to restore the primary antibody response of X-irradiated rats to diphtheria toxoid: The primary antibody response of normal adult male rats to diphtheria toxoid is shown in Figure 4. The response was abolished by exposure to 500 r whole-body irradiation 24 hours prior to the toxoid injection. Thoracic duct cells were unable to restore the response in doses as high as 1×10^9

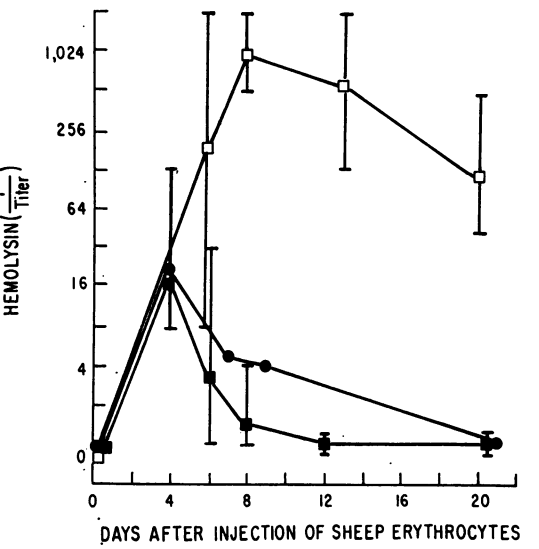

FIG. 2.—Hemolysin response to a single intravenous injection of 1×10^8 sheep red blood cells.

●—●, Mean response of ten neonatally thymectomized rats at 4 weeks of age.

□—□, Mean response of eight neonatally thymectomized rats given an intravenous injection of 2×10^8 thoracic duct cells 22 hr before injection of sheep red blood cells. Brackets show range of titers.

■—■, Mean response of eight neonatally thymectomized rats given an intravenous injection of 2×10^8 "tolerant" thoracic duct cells (obtained from rats made neonatally tolerant to sheep red blood cells) 22 hr before injection of sheep red blood cells. Brackets show range of titers.

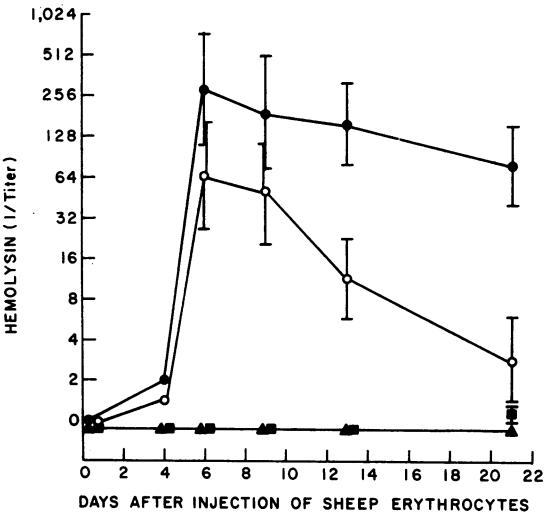

FIG. 3.—Hemolysin response of rats exposed to 500 r whole-body irradiation and given an intravenous injection of lymphoid cells 2 hr later. An intravenous injection of 1×10^8 sheep red blood cells was given 24 hr after irradiation. Brackets show standard error of the mean.

○—○, Mean response of six rats given 1×10^8 thoracic duct cells alone.

■—■, Mean response of six rats given 1×10^8 bone-marrow cells alone.

●—●, Mean response of six rats given a combination of 1×10^8 thoracic duct cells and 1×10^8 bone-marrow cells.

▲—▲, Mean response of six rats given no lymphoid cells.

cells (Fig. 4). Nevertheless, 1×10^8 spleen cells produced a demonstrable antibody response (Fig. 4). Spleen cells and thoracic duct cells were obtained from normal adult male rats.

The inactivity of thoracic duct cells may have been due to the paucity of macrophages found in thoracic duct lymph. Accordingly, several experiments were performed to determine whether the addition of macrophages to thoracic duct cells would result in restoration of the antibody response. An inoculum of 5×10^7 peritoneal exudate cells, collected from normal rats 18 hours after the intraperitoneal injection of 1 ml Bayol *F*, was injected together with 1×10^8 thoracic duct cells into irradiated hosts. In similar experiments, 1×10^8 spleen cells, obtained from rats depleted of thoracic duct cells by five days of thoracic

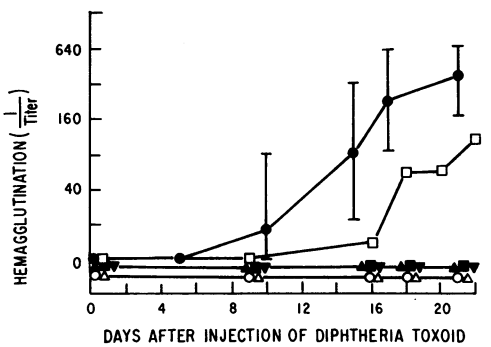

FIG. 4.—Tanned red cell hemagglutination response to a single intraperitoneal and subcutaneous injection of 15 Lf (total) alum-precipitated diphtheria toxoid. Rats exposed to 500 r whole-body irradiation were injected with diphtheria toxoid 24 hr later.

●—●, Mean response of eight normal rats. Brackets show range of titers.

▲—▲, Mean response of eight rats exposed to 500 r whole-body irradiation.

■—■, Mean response of eight rats given an intravenous injection of 2.5×10^8 thoracic duct cells 2 hr after irradiation.

▼—▼, Mean response of three rats given an intravenous injection of 1×10^9 thoracic duct cells 2 hr after irradiation.

○—○, Mean response of six rats given an intravenous injection of a combination of 5×10^7 peritoneal exudate cells and 1×10^8 thoracic duct cells 2 hr after irradiation.

△—△, Mean response of four rats given an intravenous injection of a combination of 1×10^8 spleen cells, obtained from rats depleted of thoracic duct cells by 5 days of thoracic duct drainage, and 1×10^8 thoracic duct cells 2 hr after irradiation.

□—□, Mean response of six rats given an intravenous injection of 1×10^8 spleen cells, obtained from normal rats, 2 hr after irradiation.

duct drainage, were injected together with 1×10^8 thoracic duct cells into irradiated hosts. Neither combination of cells produced measurable restoration of the primary antibody response (Fig. 4).

Restoration of the primary antibody response of neonatally thymectomized rats to diphtheria toxoid with thoracic duct cells: Figure 5 shows that 9 out of 12 neonatally thymectomized Lewis rats had no measurable antibody response following the injection of diphtheria toxoid at 10–12 weeks of age. However, 10 out of 11 thymectomized rats reconstituted with 2×10^8 thoracic duct cells 22 hours prior to the toxoid injection showed normal or above-normal antibody titers. The difference in the fraction of responders in both groups is highly significant ($p < 0.001$).

Lack of synergism between thoracic duct cells and other lymphoid cells: Although synergy between thoracic duct cells and bone-marrow cells was easily demonstrated with sheep erythrocytes as antigen, it could not be demonstrated with diphtheria toxoid. Figure 6 shows the inability of a combination of 1×10^8 bone-marrow cells and 1×10^8 thoracic duct cells to restore the antibody response of irradiated hosts. In further attempts to detect synergy, irradiated hosts were reconstituted with a combination of 1×10^8 thoracic duct cells obtained from normal donors and 1×10^8 spleen cells obtained from neonatally thymectomized donors. This combination of cells also failed to restore a measurable antibody response (Fig. 6).

Discussion.—The ability of thoracic duct cells to restore the primary hemolysin response of lethally irradiated rats indicates that this population of cells contains immunologically competent *units*. A *unit* may be defined, in the sense proposed by Medawar,[11] as a single cell or minimum combination of different cells which can restore the ability of an immunologically inert host to produce an

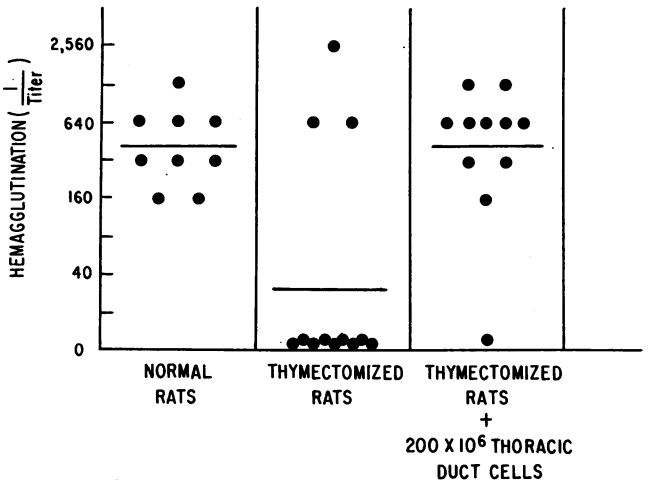

FIG. 5.—Tanned red cell hemagglutination response 21 days after a single intraperitoneal and subcutaneous injection of 15 Lf (total) alum-precipitated diphtheria toxoid. Thoracic duct cells were given intravenously 22 hr before the injection of diphtheria toxoid.

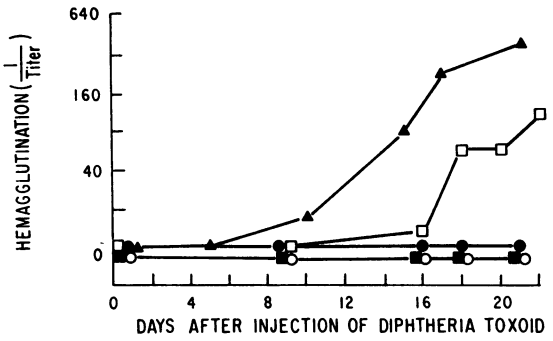

FIG. 6.—Tanned red cell hemagglutination response of rats exposed to 500 r whole-body irradiation and given an intravenous injection of lymphoid cells 2 hr later. A single intraperitoneal and subcutaneous injection of 15 Lf (total) alumprecipitated diphtheria toxoid was given 24 hr after irradiation.

●—●, Mean response of six rats given a combination of 1×10^8 thoracic duct cells and 1×10^8 bone-marrow cells.

○—○, Mean response of six rats given 1×10^8 spleen cells obtained from neonatally thymectomized donors.

■—■, Mean response of six rats given a combination of 1×10^8 spleen cells obtained from neonatally thymectomized donors and 1×10^8 thoracic duct cells obtained from normal donors.

□—□, Mean response of six rats given 1×10^8 spleen cells obtained from normal donors.

▲—▲, Mean response of eight normal rats is shown for comparison.

immune response to a given immunogen. The work of Gowans and his colleagues[9, 12] with rats and Nossal *et al.*[13] with mice strongly suggests that the immunologically competent *unit* in the sheep red blood cell system is composed exclusively of circulating small lymphocytes.

In the present study, neonatally thymectomized rats showed a marked depression of the primary hemolysin response at four weeks of age. These findings differ from those reported previously by Pinnas and Fitch.[14] These investigators found no depression of the hemagglutinin response in neonatally thymectomized rats challenged with sheep erythrocytes at 4 weeks of age and at 16 weeks of age. Perhaps differences in the antibody assay or strain of rat used in each case account for the different findings. Thoracic duct cells obtained from normal donors were able to restore the depressed response of neonatally thymectomized Lewis rats. Thoracic duct cells obtained from donors made neonatally tolerant to sheep erythrocytes were unable to restore the response. The latter observation suggests that donor cells must be able to react with the immunogenic stimulus in order to transfer immunological competence to the host.

The ability of thoracic duct cells to synergize with bone-marrow cells was demonstrated in rats. Recent evidence reported by Mitchell and Miller[10] and Nossal *et al.*[15] shows that the interaction of these cells in irradiated mice challenged with sheep erythrocytes produces antibody-forming cells derived from both cell types. However, in both rats and mice the bone-marrow cell which interacts with thoracic duct cells may itself be a member of the pool of circulating lymphocytes. Indeed, the bone-marrow cell may be identical to a cell type present in considerable numbers in thoracic duct lymph. This is not an unlikely possibility in view of the fact that, on a strictly numerical basis, thoracic duct cells alone are as efficient as an equal number of combined thoracic duct cells and bone-marrow cells in restoring the primary hemolysin response of irradiated rats (Figs. 1 and 3) or mice.[10]

Studies with diphtheria toxoid show that thoracic duct cells are unable to re-

store the primary antibody response of irradiated rats in doses as high as 1×10^9 cells. It is unlikely that this inability is a consequence of radiation damage to the macrophages of the host, for the addition of peritoneal exudate macrophages or splenic macrophages to thoracic duct cell inocula did not restore the primary response. Spleen cells were able to restore a demonstrable antibody response in irradiated hosts. The most likely explanation of these findings is that for the diphtheria toxoid system, splenic lymphoid cells contain immunologically competent *units* and thoracic duct lymphocytes do not.

Unexpectedly, thoracic duct cells did restore the primary response to diphtheria toxoid in neonatally thymectomized hosts. However, these cells did not synergize with bone-marrow cells obtained from normal donors or with spleen cells obtained from neonatally thymectomized donors. A striking similarity exists between the function of thymus cells in the sheep red blood cell system in mice, reported by Mitchell and Miller,[10, 16] and that of thoracic duct cells in the diphtheria toxoid system in rats. Both cells are able to restore the antibody response of thymectomized hosts but not that of irradiated hosts, and both show little ability to synergize with other lymphoid cells in irradiated hosts.

In conclusion, the experimental results show that the cells involved in the initiation of the primary antibody response to sheep erythrocytes differ from those involved in the response to diphtheria toxoid. In the sheep erythrocyte system, circulating lymphocytes make up the immunologically competent *unit*, and synergy between bone-marrow and thoracic duct cells may represent the interaction between two different classes of circulating cells. In the diphtheria toxoid system, immunologically competent *units* can be formed from splenic lymphoid cells. Circulating lymphocytes are probably not part of this *unit*, but experiments with neonatally thymectomized hosts suggest that the circulating cells may eventually differentiate into other cells which are part of the *unit*.

Summary.—Thoracic duct cells restored the primary antibody response of X-irradiated and neonatally thymectomized rats challenged with sheep red blood cells. Synergy between thoracic duct cells and bone-marrow cells was demonstrated in the irradiated rats. On the other hand, thoracic duct cells did not restore the primary antibody response of irradiated rats challenged with diphtheria toxoid, but did restore the response of neonatally thymectomized rats. Synergy between thoracic duct cells and bone-marrow cells obtained from normal donors, or spleen cells obtained from thymectomized donors, could not be demonstrated in the irradiated rats. It was concluded that the cellular events involved in the initiation of the primary antibody response to sheep red blood cells differ from those involved in the response to diphtheria toxoid.

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