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## Immunologic Restoration of Neonatally Thymectomized Rats With Thoracic Duct Lymphocytes. (32032)

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Previous investigations have established that neonatal thymectomy in the rat, as with other rodents, leads to immunologic deficits. While the wasting syndrome that routinely follows neonatal thymectomy in many strains of mice seems to be less common (1), neonatal thymectomy in the rat is characterized by a depletion of small lymphocytes from blood and lymphoid tissue(2), absence or depression of delayed hypersensitivity and Arthus reaction, and a decrease in production of some but not all humoral antibodies(3,4). There is also an increased survival of skin homografts when animals of minimal genetic disparity are employed as donor and host(5). Furthermore, an increased susceptibility to the induction of tumors by polyoma virus and murine sarcoma virus (Moloney strain) has been noted(6,7).

Experiments in which the neonatally thymectomized animal is "restored" with either an injection of a certain cell type or graft of a specific organ have been carried out to ascertain information concerning both the nature of the deficit of the thymectomized animal and the function of donor tissue. Restoration experiments have shown that the immunologic deficit incurred following thymectomy can be reversed either by restoring to the animal the factors necessary for its own immune system to mature to a state of immunological competence or by re-equipping the animal with immunologically competent cells taken from normal adult syngeneic donors.

The latter type of experiment in which thymectomized animals have been re-equipped or reconstituted with immunologically competent donor lymphoid tissue have been performed using either spleen or lymph nodes as the source of cells. Because of the heterogeneous population of cells in the spleen and lymph node it has not been possible to say with exactness the actual cell involved in these restoration experiments. Cannulation of the thoracic duct of the rat yields a morphologically relatively homogeneous population of cells, consisting of greater than 95% small lymphocytes and the residual large lymphocytes(15). It seemed of interest, therefore, to test the ability of thoracic duct cells to restore the immunological deficits of the rat.

Three immunological responses normally present in the adult rat but absent or severely reduced in the neonatally thymectomized animal have been used to test the restorative ability of the thoracic duct cells. These immune responses are the capacity to undergo an Arthus and delayed hypersensitivity reaction under the proper stimulus and the ability to prevent development of tumor growth following inoculation with polyoma virus during the first week after birth. That the latter phenomenon does have an immunological basis seems to be reasonably well established(16). This is discussed later.

The present report describes attempts to restore neonatally thymectomized rats to immunological competence with thoracic duct

lymphocytes. A comparison is made of the restorative capacity between an inoculum relatively rich in large lymphocytes and an inoculum made up largely of small lymphocytes. The restorative ability of lymphocytes obtained from the thoracic ducts of neonatally thymectomized rats has also been tested. The results obtained show that the immune responses of thymectomized rats can be restored by thoracic duct cells, and that restoration is correlated with the number of small lymphocytes in the inoculum but not with the number of large lymphocytes. Finally, lymphocytes obtained from neonatally thymectomized donors show no restorative effect even when given in high doses.

*Materials and methods. Rats.* Inbred Lewis strain rats obtained from Microbiological Associates, Walkersville, Md. were used.

*Virus.* Standardized large plaque polyoma virus derived from the LID-1 strain was used throughout(17). Virus was grown on mouse embryo cells. A standard inoculum of between one and three million plaque forming units (PFU) in 0.1 cc total fluid was injected into the right axillary region of each animal at 6 to 8 days of age. Each animal was inspected and palpated at weekly intervals beginning at 6 weeks of age and the time at which a tumor was first seen or palpated was noted.

*Thymectomy.* Thymectomy of rats was performed within the first 72 hours of birth by a method adapted from Miller(18). Thymectomized donors of thoracic duct lymphocytes were thymectomized within 24 hours of birth as were a great majority of the animals used in the experiment. All animals tested for delayed hypersensitivity were thymectomized within 24 hours of birth. At the end of 6 months or at the time a tumor was first noted, animals were routinely autopsied, the superior mediastinum checked for residual thymus and serial sections studied where indicated. Thymectomized animals found to have residual thymus were excluded from the results.

*Restoration procedures.* Thoracic Duct Lymphocytes (TDL) were obtained from male animals weighing 200-250 g. The thoracic duct was cannulated according to the method of Wilson(19). Lymph was collected for 24-hour periods in Ringer's solution containing heparin sodium, 50 units per cubic milliliter, and maintained between 4° and 10°C. The cells were concentrated by centrifugation, resuspended in Ringer's, counted, and tested for viability by staining with 0.16% trypan blue for 3 minutes. Those cells that did not take up stain were considered viable. Giemsa smears were performed on most aliquots for differential counts.

Spleen cells from normal intact Lewis rats weighing 200 to 250 g were prepared according to the method of Billingham(20). Viability counts were performed as above.

Injections of cells were performed 2 to 4 days after thymectomy. Animals were given 0.1 cc of fluid containing cells in the orbital branch of the anterior facial vein.

*Delayed hypersensitivity.* Animals were inoculated at 10 to 14 weeks of age with an emulsion containing bovine serum albumin (BSA) in complete Freund's adjuvant. Each animal received 0.1 cc emulsion in each hind foot pad for a total dose of 700 mg to 800 mg protein per animal. The animals were skin tested 12 to 14 days following immunization with an intradermal injection of 50 $\mu$g BSA in 0.1 cc saline. Skin reactions were read at 4 hours (Arthus) and 24 hours (delayed hypersensitivity) and scored according to their diameter and thickness of induration. Animals in which no induration was palpable were scored as 0. There were a few animals in which induration was obviously present but the boundaries were too indiscrete to allow a meaningful measurement. These were scored as +1. In all cases the diameter of the area of induration was at least 7 mm. In reactions of greater magnitude, the diameters were measured and the thickness of induration graded subjectively on a scale of 2+ to 3+. It should be noted that all restored animals and nonthymectomized control animals tested for delayed hypersensitivity had also been given polyoma virus. Several thymectomized animals not given virus were used as controls. These animals were similar to their counterparts in every other respect. These controls

TABLE I. Response of Thymectomized, Non-Thymectomized and Restored Lewis Strain Rats to LID-1 Polyoma Virus.

| Treatment | No. rats with tumors/No. inoculated | Percentage rats with tumors | Mean latent period‡ (wk) |
|---|---|---|---|
| Thymectomized | 64/65 | 98 | 9.4 ( 7–17)§ |
| Non-thymectomized | 4/51 | 8 | 17.3 (13–26) |
| Thymectomized + $1 \times 10^6$ TDL* | 9/ 9 | 100 | 10.7 ( 8–13) |
| " + $5 \times 10^6$ TDL | 6/ 8 | 75 | 12.7 ( 8–19) |
| " + $10 \times 10^6$ TDL | 16/24 | 67 | 14.4 ( 7–26) |
| " + $20 \times 10^6$ TDL | 8/23 | 35 | 17.2 ( 7–26) |
| " + $10 \times 10^6$ PDTDL† | 15/17 | 89 | 10.8 ( 8–18) |
| " + $20 \times 10^6$ spleen cells | 12/15 | 80 | 14.6 ( 9–26) |

* TDL—Thoracic duct lymphocytes containing greater than 95% small lymphocytes.
† PDTDL—Prolonged drainage TDL, containing 20-30% large lymphocytes.
‡ Latent period refers to time between virus inoculation and time tumor first palpated.
§ Numbers in parentheses refer to range in weeks at which tumor appeared.

were necessary due to the high incidence of tumor induction in the non-restored thymectomized animals.

*Histology.* All animals were autopsied when they became tumor positive and sections of spleen, cervical lymph nodes, and, occasionally, tumor were fixed in Tellyesniczky's fluid and stained with hematoxylin and eosin.

*Lymphocyte counts.* Animals at age 2 months were bled from the tail vein. The total white count and percentage of lymphocytes was determined and a calculation of total peripheral lymphocytes made.

*Results. Protection from virus induced tumors.* Lewis rats thymectomized within 72 hours after birth quickly recovered from surgery, appeared normal and grew at a rate similar to nonthymectomized controls. There was no evidence of wasting, but young adult thymectomized animals seemed more susceptible to laboratory infections and had a higher death rate than control animals.

Thymectomized rats injected subcutaneously with polyoma virus in the stated dose at one week of age almost invariably developed subcutaneous neoplasms usually at or near the site of injection. Not infrequently multiple subcutaneous tumors arose simultaneously. Rarely, solitary subcutaneous neoplasms developed at distant sites. The only other location of tumor growth was in one or both kidneys and this occurred in less than 3% of the tumor-positive animals. All animals with renal neoplasms also had one or more subcutaneous tumors. All tumors grew progressively without signs of regression. The histology of the tumors sampled, subcutaneous or renal, was similar and was consistent with a well differentiated fibrosarcoma.

Tumors that arose in animals reconstituted with various cell preparations or in nonthymectomized control rats were similar to tumors in thymectomized animals in respect to location, appearance, histology and growth patterns. There was, however, a difference between the 3 general groups of animals, thymectomized alone, nonoperated controls and thymectomized plus reconstitution, in terms of percentages of animals developing tumor and the latent period between virus inoculation and tumor growth.

Table I shows the percentage of animals developing tumors and the mean latent period between virus inoculation and tumor growth for the respective groups of animals. Ninety-eight percent of thymectomized animals developed tumors (64/65). The earliest tumors were seen 7 weeks after viral inoculation and 95% of animals were tumorous by 13 weeks, the mean latent period being 9.4 weeks. Fifty-five percent of thymectomized animals developed tumors during the ninth and tenth week after viral inoculation. In contrast were the nonthymectomized control rats. Only 4 of these 51 animals, or 8% developed tumors in the 6-month experimental period. The latent period was also considerably longer, 17.3 weeks, in this group of animals.

Reconstitution of thymectomized animals

was attempted with several different cell populations. Inocula of thoracic duct lymphocytes, containing between 95% and 98% small lymphocytes, comprised the first population of cells tested. Note (Table I) that as the number of injected lymphocytes per animal increased, both the percentage of animals protected from tumor growth and the latent period between viral challenge and tumor development increased. One million cells essentially had no effect in preventing tumor growth. The group of animals receiving 5 million cells was rather small, but there was an indication that a small percentage of thymectomized animals were being protected from tumor growth even with this dose. With a dose of 10 million thoracic duct cells per animal protection from tumor growth was increased, and with 20 million cells the incidence of animals developing tumors was reduced to 33%. Moreover, the mean latent period became longer as the dose of cells was augmented, increasing from 9.4 weeks for a thymectomized animal to 14.4 weeks for an animal receiving 10 million TDL and to 17 weeks for a rat receiving 20 million cells.

The inoculum of thoracic duct cells used in the above experiments contained up to 5% large lymphocytes along with the small lymphocytes. To test the importance of the size of lymphocytes *per se* on the ability to reconstitute thymectomized rats, another group of animals was injected with an inoculum of lymphocytes collected on the sixth, seventh or eighth day following cannulation. In this inoculum large lymphocytes comprised 20 to 30% of the total cell population and, therefore, represented an increase of at least 4 to 6 times the number of large lymphocytes present in the previous experiment. Stated another way, an inoculum of 10 million TDL collected one week following cannulation contained more large lymphocytes than a dose of 40 to 60 million cells collected immediately after cannulation. It can be seen in Table I that despite this increase in the number of large lymphocytes, the protection afforded against tumor growth was less than with a comparable number of small lymphocytes. Thus, an increase in the number of large lymphocytes *per se* used as donor cells was not correlated with an increase in protection.

TABLE II. Output of Thoracic Duct Lymphocytes Over a 72 Hr Period. Normal *vs* neonatally thymectomized donors.

| No. | Treatment | TDL output ($\times 10^6$) 1-24 hr | 24-48 hr | 48-72 hr | Total TDL 72 hr |
|---|---|---|---|---|---|
| 1 | Thymectomized | 157 | 72 | 105 | 334 |
| 2 | " | 110 | 143 | 75 | 328 |
| 3 | " | 60 | 158 | 125 | 343 |
| 4 | " | 112 | 130 | 75 | 317 |
| 5 | " | 110 | 40 | 22 | 172 |
| 6 | " | 285 | 100 | 100 | 485 |
| | Avg | 139 | 107 | 84 | 330 |
| 7 | Normal | 260 | 427 | 214 | 901 |
| 8 | " | 270 | 425 | 334 | 1029 |
| 9 | " | 460 | 386 | 208 | 1054 |
| 10 | " | 273 | 519 | 280 | 1062 |
| 11 | " | 480 | 320 | 200 | 1050 |
| | Avg | 349 | 415 | 247 | 1019 |

Another group of thymectomized animals was restored with 20 million syngeneic spleen cells. This inoculum contained a relatively high number of large lymphocytes, plasma cells and macrophages. This number of spleen cells, although considerably less effective in protecting rats from tumor growth than an equivalent number of TDL, did cause a significant prolongation of the mean latent period and a small decrease in the percentage of animals developing tumors.

Table II compares the total output of lymphocytes over a 72-hour period following cannulation of the thoracic duct of a group of rats thymectomized at birth and non-thymectomized rats of comparable age and weight. Several of the control rats were litter mates of the thymectomized animals. Normal rats drained approximately 3 times the total number of cells over this period.

To determine the ability of the TDL from thymectomized donors to protect against tumor growth in thymectomized syngeneic hosts, inocula ranging from 10 to 40 million TDL from thymectomized rats were injected as in the experiments above. Although the percentage of small lymphocytes in these inocula was slightly lower than in normal animals, small lymphocytes still made up more than 90% of the cell population. In Table

TABLE III. Response of Thymectomized Lewis Strain Rats "Restored" with TDL from Thymectomized Donors to LID-1 Polyoma Virus.

| Treatment | No. rats with tumors/No. inoculated | Percentage rats with tumors | Mean latent period (wk) |
|---|---|---|---|
| Thymectomized | 64/65 | 98 | 9.4 (7–17) |
| " + 10 × $10^6$ TTDL* | 10/10 | 100 | 8.5 (7–10) |
| " + 20 × $10^6$ TTDL | 11/12 | 92 | 9.3 (9–11) |
| " + 40 × $10^6$ TTDL | 5/ 5 | 100 | 10.8 (9–14) |
| Combination thymectomized + 10-40 × $10^6$ TDL | 26/27 | 96 | 9.6 (7–14) |

* TTDL—Thymectomized thoracic duct lymphocytes (*i.e.*, TDL from thymectomized donor).

III it is seen that the percentage of tumorous animals as well as the latent period between viral inoculation and tumor development in those thymectomized rats given TDL from thymectomized donors, even in doses of 40 million lymphocytes, were essentially the same as in animals thymectomized only. Although there may have been a slight prolongation of the latent period as the cell dose was increased from 10 million to 40 million cells, the degree of protection offered by these cells was negligible.

*Delayed hypersensitivity and Arthus reactions.* A representative number of animals from groups of thymectomized rats, nonthymectomized controls and thymectomized rats receiving various doses of TDL were tested for delayed hypersensitivity and Arthus reactions to BSA. These animals, except for several of the thymectomized control animals, were the same animals used in the previous experiment. They were immunized at 10 to 14 weeks of age and skin tested approximately 2 weeks later. Table IV lists the results. Normal animals invariably gave an excellent response and thymectomized animals showed no reaction or, as in one case, an extremely weak reaction at 24 hours. The responses of the reconstituted animals were somewhat variable. About one-half of the rats restored with 10 million TDL showed positive delayed hypersensitivity and Arthus reactions, and all 10 animals given 20 million TDL were positive for delayed hypersensitivity, 7 of these also showing Arthus reactions. The reactions in many of these animals were much less than those elicited in nonthymectomized controls, but all were definitely positive as compared with thymectomized controls.

Many animals showing a positive delayed hypersensitivity and Arthus reactions subsequently developed tumors. On the other hand, of the animals not developing tumors, all but one tested also had a positive delayed hypersensitivity response. There seemed to be no strict correlation between positive Arthus reactions and delayed hypersensitivity. A majority of the animals positive for both reactions had greater Arthus reactions than delayed hypersensitivity.

*Peripheral lymphocyte counts and histological findings.* Peripheral lymphocyte counts from a representative number of thymectomized rats restored with 20 million TDL, thymectomized only rats and nonthymectomized control rats were performed at approximately 2 months of age. While there was a moderate amount of variation within each group the average number of lymphocytes for both groups of thymectomized animals, restored and nonrestored, was almost identical, being about 3300 lymphocytes per/$mm^3$. This compared with an average of 6700/$mm^3$ for the nonthymectomized controls. Of the thymectomized animals there was more variation between different litters than there was between the different groups of restored and nonrestored animals.

Rats were sacrificed at the time they first became tumor positive or at 6 months of age at the conclusion of the experiment. Histological sections were made of cervical lymph nodes and spleen. Again there was a wide variation between nonthymectomized control rats and thymectomized animals. The latter showed slight to marked depletion of small lymphocytes from lymph nodes and the splenic white pulp. Those areas normally occupied by small lymphocytes contained larger cells

with larger pale staining nuclei, presumably reticular cells. There were far fewer primary follicles and in many lymph nodes no follicles could be seen. As with peripheral lymphocyte counts very little difference could be discerned between thymectomized animals restored with 20 million lymphocytes shortly after birth and animals thymectomized alone.

TABLE IV. Delayed Hypersensitivity and Arthus Reactions in Thymectomized Rats Restored at Birth with Thoracic Duct Lymphocytes.

| Thymectomized | | Thymectomized + $10 \times 10^6$ TDL | | Thymectomized + $20 \times 10^6$ TDL | | | Control | |
|---|---|---|---|---|---|---|---|---|
| Sex | 4 hr | 24 hr | Sex | 4 hr | 24 hr | Sex | 4 hr | 24 hr |
| ♀ | 0 | (1+) | ♀ | 0 | (1+) | ♀ | 19mm(3+) | 12mm(2+) |
| ♀ | 0 | 0 | ♀ | 0 | (1+) | ♀ | 18mm(3+) | 13mm(2+) |
| ♀ | 0 | 0 | ♀ | 0 | (1+) | ♀ | 17mm(3+) | 14mm(3+) |
| ♀ | 0 | 0 | ♀ | 18(3+) | 12(2+) | ♀ | 17mm(3+) | 15mm(3+) |
| ♀ | 0 | 0 | ♀ | (1+) | 13(2+) | ♀ | 20mm(3+) | 15mm(3+) |
| ♀ | 0 | 0 | ♀ | 19mm(3+) | 14mm(3+) | ♀ | 10mm(2+) | 17mm(3+) |
| ♀ | 0 | 0 | ♀ | 18mm(3+) | 16mm(3+) | ♀ | 14mm(3+) | 19mm(3+) |
| ♀ | 0 | 0 | ♀ | 20mm(3+) | 15mm(2+) | | | |
| ♀ | 0 | 0 | ♀ | 16mm(3+) | 16mm(3+) | | | |
| | | | ♀ | 15mm(2+) | 17mm(3+) | | | |
| | | 12mm(2+) | | 0 | | | | |
| | | 20mm(3+) | | 12mm(2+) | | | | |
| | | (1+) | | (1+) | | | | |
| | | 18mm(2+) | | 12mm(2+) | | | | |
| | | 12mm(2+) | | 14mm(3+) | | | | |
| | | 0 | | 12mm(2+) | | | | |
| | | 18mm(3+) | | 15mm(2+) | | | | |
| | | 0 | | (1+) | | | | |
| | | 20mm(3+) | | 15(3+) | | | | |
| | | | | Total positive/Total challenged | | | | |
| 0/9 | 1/9 | 7/14 | | 8/14 | 7/10 | 10/10 | 7/7 | 7/7 |

While some lymph nodes and spleens appeared less depleted than others, none of the thymectomized animals approached normal histological appearance whether they had received lymphocytes or not. Moreover, there was no correlation noted between degrees of depletion and immunological reactivity among the thymectomized only animals and the restored thymectomized animals.

*Discussion.* These experiments demonstrate that thoracic duct lymphocytes are capable of reconstituting the immune system of neonatally thymectomized rats to the degree that a high percentage of animals are protected from tumor induction subsequent to an inoculation with polyoma virus at one week of age. Thymectomized animals receiving TDL also demonstrate positive, albeit somewhat less than normal, delayed hypersensitivity reactions and Arthus reactions. The degree of restoration is proportional to the number of cells infused. The protective effect against tumor oncogenesis is greater when an inoculum containing mostly small lymphocytes is used compared with an inoculum containing a greater proportion of large lymphocytes. This latter finding suggests that the effective cell in restoring the thymectomized animal is the circulating small lymphocyte. More evidence to this effect is provided by the fact that an inoculum of spleen cells, containing many more large lymphocytes, macrophages, and plasma cells is less effective than an equivalent dose of thoracic duct lymphocytes. Alternatively, the large lymphocytes found in lymph immediately following cannulation may represent a different, more immunocompetent, cell population than large lymphocytes found in the lymph draining several days after cannulation and in the spleen. Morphologically there seems to be no difference, but further evidence is needed if this latter possibility is to be ruled out. Experiments in which the number of large lymphocytes is reduced below one percent by incubation of the cells are presently being carried out and should help answer the question.

The importance of the thoracic duct lymphocyte and particularly the small lymphocyte has been established in many immune

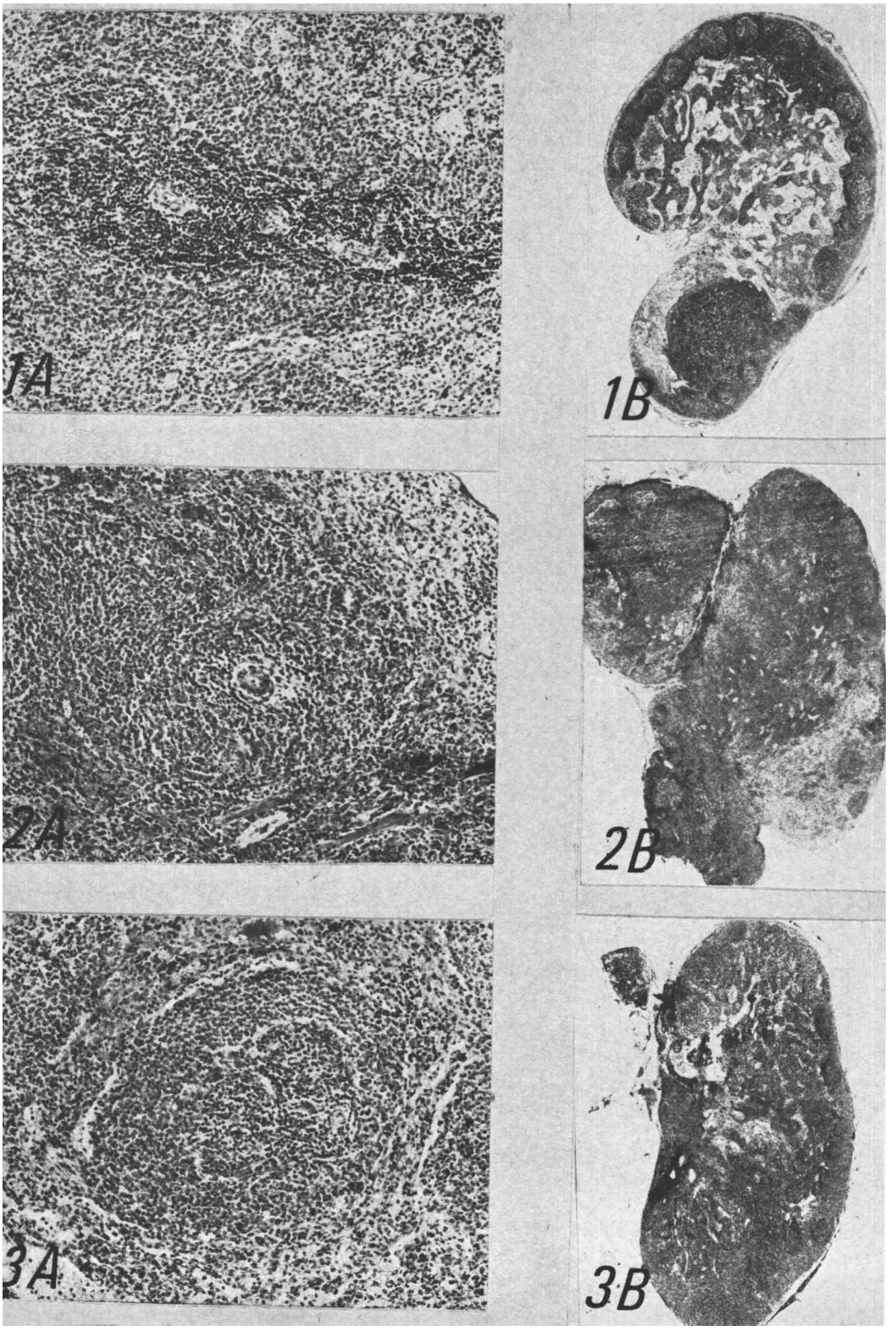
1A
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FIG. 1-3 A) White pulp of spleen ×160. B) Lymph node ×30.

FIG. 1. Non-thymectomized control rat, sacrificed at age 6 mos.

FIG. 2. Thymectomized rat which developed tumor and was sacificed at age 10 wks. Note depletion of darkly staining small lymphocytes from white pulp of spleen, primary follicles and medullary cords of lymph nodes and replacement with reticular cells.

FIG. 3. Thymectomized rat receiving $20 \times 10^6$ TDL at age 3 days and immunologically restored. Note similarity to Fig. 2. Rat sacificed at age 6 mos.

responses including homograft rejection(21), graft *versus* host reaction(15) and the production of certain circulating antibodies(22). The significance of these cells in the oncogenesis of polyoma viral induced tumors and the delayed hypersensitivity reaction seems evident from the above experiments.

It would seem most plausible that the protection afforded by TDL injected into neonatally thymectomized rats given polyoma virus is mediated by a homograft-type response directed against a tumor specific antigen present in the neoplastic cell. The evidence for believing that polyoma virus tumor induction is directly related to the immune status of the host animal has been recently reviewed(16). This evidence may be summarized as follows: First, neoplasms developing subsequent to inoculation of polyoma virus contain new tumor specific antigens(23,24). Thus, immunization of adult mice or rats with either polyoma virus, or by virus-free tumor containing the specific polyoma tumor antigen, will protect the host animal when subsequently challenged with a syngeneic polyoma tumor(23,26,27). Second, neonatal thymectomy which leads to a state of immunologic deficiency, particularly of the homograft type response, strikingly increases the incidence of tumors in animals injected at a young age with polyoma virus(6,25). Third, the increased incidence of tumors in thymectomized mice can be prevented by the measures that are known from other test systems to restore, at least partially, the animals' immunologic competence. These measures include grafts of syngeneic lymphoid tissue from the spleen, whole thymus grafts and thymus grafts placed intraperitoneally in cell impermeable Millipore chambers(16). Passive transfer of serum from animals immune to polyoma virus-induced tumor cells is ineffective in protecting neonatally thymectomized animals from polyoma virus-induced neoplasms(28). Fourth, the presence or absence of thymic tissue has no effect on either the virus growth pattern or on the virus-induced circulating antibodies which are similar in thymectomized and intact mice(16). Thus, the susceptibility of neonatally thymectomized mice to tumor induction by polyoma virus is not due to either a difference in virus growth or a decrease in humoral antibodies directed against the virus. While these arguments are not conclusive, they strongly support the concept that the immune status of the host animal is an important factor in determining whether an animal infected with polyoma virus will or will not develop a tumor. In particular, the type of immunity involved seems to be the cellular mediated homograft type reaction. According to this concept neoplastic changes occur in cells infected with polyoma virus. These neoplastic cells contain the tumor specific antigen which in the normal animal is recognized as "foreign." A homograft type of response directed against the neoplastic cells ensues and the cells are eliminated, thus preventing tumor growth. The thymetomized animal, having a deficient immune system, is unable to mount an effective homograft rejection response. The neoplastic cells are not destroyed and tumor growth progresses.

The findings in this report are consistent with this hypothesis. It has been amply demonstrated that thoracic duct lymphocytes and particularly small lymphocytes are important in the cellular mediated homograft-type immune response. It seems likely that a fraction of the donor lymphocytes injected at birth into neonatally thymectomized host, or the progeny of these cells, continues to function normally in the host animal and thereby reconstitutes the animal's immune system. As viral induced neoplastic cells develop, the reconstituted animal is able to mount an effective homograft rejection response and destroy the neoplastic cells.

It seems significant that these same animals are able to undergo positive delayed

hypersensitivity and Arthus reactions. Isakovic *et al* demonstrated that the immune deficit present in thymectomized animals which prevents positive delayed hypersensitivity and Arthus responses can be overcome with an infusion of living lymph node cells given at the time of immunization, 10 weeks after birth(14). Our experiments differ from Isakovic's in several respects. We injected far fewer cells and the cells originated from the thoracic duct. The cells were injected at birth and the animals were not immunized until 3 to 4 months later. The response, although much greater than in thymectomized controls, was often less than that seen in normal animals. The relevance of Isakovic's work to our findings is to show that living lymph node cells will reconstitute the thymectomized animal. The fact that the rats in our experiment showed a positive delayed hypersensitivity and Arthus responses is strongly suggestive that some of the donor cells or their progeny are still living and functioning adequately at least 3 to 4 months after injection. It does not seem unreasonable, therefore, to hypothesize that there are also cells living and capable of reconstituting the rat's immune mechanism in order that it can mount an effective homograft type reaction against neoplastic cells containing polyoma tumor specific antigen.

Although these experiments demonstrate that the homogeneous population of lymphocytes from the thoracic duct is capable of reconstituting to some extent the immunological capacity of neonatally thymectomized rats, they give very little direct information as to the actual mechanisms of these responses except to emphasize the essentiality of lymphocytes or progeny of lymphocytes in some stage of these responses. Moreover, it cannot be stated whether the cells actually responsible for this restoring effect are the long lived small lymphocytes known to be present in the thoracic duct or progeny of dividing cells. It seems clear, however, whichever type of cells is involved, that once a lymphocyte achieves immunological competence under the influence of the thymus it can function normally and for a prolonged period of time in a milieu devoid of the thymus gland.

The findings reported here that the number of thoracic duct lymphocytes from neonatally thymectomized donors is considerably less than normal and that in addition these cells are completely without effect in preventing polyoma-induced tumor growth in neonatally thymectomized syngeneic hosts demonstrates the qualitative and quantitative deficiency in the population of lymphocytes in a thymectomized animal. The data pertaining to the number of lymphocytes collected over a 72-hour period are consistent with the previous findings of Schooley *et al* that in the first 2 or 3 hours following cannulation, the thoracic duct lymphocyte output of rats thymectomized at 6-8 days was about 27% of that found in sham-treated controls(4). We can extend this finding and say that this ratio continues over a more prolonged period of drainage.

Rieke has recently demonstrated, and we have confirmed, the inability of thoracic duct lymphocytes from thymectomized Lewis donors to cause a graft *versus* host (GVH) reaction in newborn BN rats, even when given in doses up to 10 times the minimum number of normal thoracic duct lymphocytes required to cause a lethal GVH reaction(29). It seems likely that both the experiments reported above and Rieke's GVH experiments reflect a dearth of cells capable of carrying out a homograft type immune response in the lymphocyte population of the thymectomized host. Accompanying the marked reduction in number of lymphocytes in a thymectomized animal, there is a further selective deficit of cells able to carry out certain immune responses.

Finally, in thymectomized rats given 20 million TDL shortly after birth there is no evidence of histological restoration although these animals are immunologically restored. This does not seem surprising. The total complement of circulation small lymphocytes in a normal adult rat has been estimated to be approximately two billion cells(30). From the number of lymphocytes that are found in the blood and thoracic duct of thymectomized rats, they appear to have approximately 30% of the normal number of circulating lymphocytes. Twenty million

lymphocytes even if they should divide several times would probably not make a measurable difference in the total number of circulating lymphocytes in the thymectomized animal or in the histological appearance of the lymph node. Osoba has noted that in certain strains of mice neonatally thymectomized and restored with thymus grafts contained in cell impermeable Millipore chambers, immunologic restoration is not accompanied by histological restoration(31). It would seem from Osoba's and our experiments that a relatively small increase in the number of competent lymphocytes in the lymphocyte pool of a thymectomized animal will restore the animal in large measure to a state of immunologic competency.

*Summary.* 1. Cells from the thoracic duct of Lewis strain rats when injected into syngeneic neonatally thymectomized rats are capable of reconstituting the host rats so that they are resistant to polyoma virus-induced tumors and can undergo positive delayed hypersensitivity reactions and Arthus reactions. 2. The protection afforded against polyoma-induced tumors is correlated with the number of small lymphocytes in the inoculum and not with the number of large lymphocytes. That this may be related to some other difference than mere size between cells found in the first day after cannulation and cells drained several days after cannulation cannot be ruled out. 3. Cells from spleens of normal Lewis rats are less effective than equal numbers of thoracic duct lymphocytes in reconstituting thymectomized rats. 4. Thoracic duct lymphocytes from neonatally thymectomized Lewis donors are not only much less numerous but also are much less effective on a per cell basis than lymphocytes from normal animals in reconstituting thymectomized hosts, thus demonstrating a qualitative as well as quantitative difference in the lymphocyte population. 5. Functional restoration is not correlated with histological restoration. 6. The possible mechanism of reconstitution is discussed.

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## Immunologic Studies in Thermal Injury: Heterophile Antibodies.* (32033)

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A previous study(1) performed on rats with experimentally induced skin burns, demonstrated formation of agglutinins directed against syngeneic erythrocytes. These hemagglutinins could be demonstrated in the thoracic duct lymph but not in blood serum. Erythrocytes of some animals gave a positive direct Coombs test. The serological findings were interpreted by postulating that the burned animals form auto-hemagglutinins which are removed by red blood cells from the blood circulation but not from the lymphatic circulation.

Attempts were also made to demonstrate formation of heterophile antibodies directed against erythrocytes of foreign species. However, such antibodies were not found in burned rats. In considering the possibility that another species may be a better subject for studies on heterophile antibodies, the present experiments were performed on guinea pigs. In addition to the search for hetero-hemagglutinins, sera of burned guinea pigs were studied for the presence of anti-gamma globulin factors resembling the rheumatoid factor. These studies were later extended to rabbits, and a few sera from human patients with severe burns were also examined.

*Materials and methods. Sera.* Non-inbred male albino guinea pigs of the Hartley strain, weighing 300 to 350 g, were purchased from Stuart Banta, Hagaman, N. Y. Thirty-nine guinea pigs were subjected to full-thickness skin burns by a procedure described previously(1,2). A thermally-regulated metal surface maintained at 250°C was applied to 20% of the body surface area of the animals under ether anesthesia. After the first burn, 27 animals received 5 additional skin burns on a 1 sq cm area at weekly intervals. The repeatedly burned guinea pigs were exsanguinated by cardiac puncture one week after the last injury. The remaining 12 animals were exsanguinated at different times from 1 to 56 days after their first and only burn.

Sequential serum samples were obtained from 4 rabbits which received 3 skin burns in a manner similar to that used for guinea pigs. The rabbits were bled before injury and one week after the first, second, and third burning.

Human sera were obtained from 5 patients suffering from severe skins burns, hospitalized at the New York University Medical Center.

*Erythrocytes.* Human and animal blood was drawn into ACD solution and preserved for not longer than one week at 4°C. Before use, the erythrocytes were washed 3 times with phosphate-buffered saline solution, pH 7.2.

*Serologic tests.* Hemagglutination tests were performed by a procedure described previouly(1). For detection of anti-human gamma globulin factors, the latex slide test (3), and a hemagglutination test with sensitized human red blood cells (SHC)(4), were

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