BONE MARROW AS SOURCE OF CELLS IN REACTIONS OF CELLULAR HYPERSENSITIVITY

I. Passive Transfer of Tuberculin Sensitivity in Syngeneic Systems*‡

By DAVID M. LUBAROFF,§ Ph.D. and BYRON H. WAKSMAN, M.D.

(From the Department of Microbiology, Yale University, New Haven, Connecticut 06510)

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Delayed hypersensitivity reactions in the skin show cellular infiltration consisting of two components: a diffuse infiltration throughout the dermis, underlying fascia, and muscle and a multifocal reaction around thin walled vessels (1, 2). The infiltrating cells have been described as lymphocytes and histiocytes (2, 3) and shown, by labeling experiments with tritiated thymidine, to come largely or entirely from a dividing cell population *via* the blood stream (3).

Evidence from a number of laboratories (4–10) indicates that the majority of infiltrating cells in delayed reactions are not actively sensitized cells. The elegant experiments of McCluskey et al. (9), in which passive transfer was combined with thymidine labeling of donor or recipient, demonstrated that 80–90% of the cells arriving in specific skin reactions are host derived. They constitute therefore a nonspecific component of the reaction. They are phagocytic and resemble the histiocytes (macrophages) appearing in sites of nonspecific inflammation (1). The latter have recently been shown to originate in the bone marrow (11), and it was of some interest to determine whether the cells appearing in delayed reactions have a similar origin. Evidence presented in the present report and in the following paper supports the hypothesis that bone marrow cells are necessary for the production of intense delayed skin reactions in the rat and include the precursors for the majority of the cells infiltrating dermal reaction sites.

Materials and Methods

Animals.—All animals were male rats of the inbred Lewis strain, obtained from Microbiological Associates, Inc. Bethesda, Md. Some were splenectomized or thymectomized at 3–4 months of age (12).

Preparation of Recipients.—Rats used as recipients were thymectomized at 5–7 wk and given 900 rads of total-body X-irradiation at 8–10 wks of age. The conditions of irradiation

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§ Present address: Department of Medical Genetics, University of Pennsylvania, Philadelphia, Pa. 19104

were: Westinghouse 250 kv machine at 15 ma using 2 mm aluminum filtration, 0.8 mm copper half-value layer, with a focus-to-skin distance of 54 cm. The dose rate was a constant 147.3 rads/min. The rats were placed in a rotating three compartment lucite cage and irradiated from above. The 900 rads was the dose of X-irradiation delivered to the middle of the animal as determined in a phantom. The inhomogeneity was ±25% which made the dose to the dorsal surface 1125 R and the dose to the ventral surface 675 R.

Transfer of Normal Cells.—Normal rats, 3–4 months of age, were killed and femoral and humeral *bone marrow* washed from the bones using Hanks' balanced salt solution (HBSS—Grand Island Biological Co., Grand Island, N.Y.). After debris was allowed to settle, the cell-rich supernatant fluid was decanted and centrifuged at 180 *g* at 4°C for 10 min. The cells were resuspended and counted, and 0.125–4.0 × 10^8 nucleated cells were injected intravenously into recipients on the day of irradiation.

Peritoneal cells were obtained from similar donors, injected intraperitoneally with 6 ml of 1.0% oyster glycogen (Eastman Chemical Products, Inc., Kingsport, Tenn.), given second and third doses of 4 ml at 48 hr intervals, and sacrificed at 6 days (13). The cells were removed from the peritoneum by washing two to three times with 50 ml of HBSS, centrifuged, resuspended, and counted. Doses of 3–4 × 10^8 nucleated cells were injected intravenously into recipient rats on the day of irradiation.

TABLE I

Effect of Splenectomy and Thymectomy on the Delayed Skin Reaction

Operative procedure	24 hr. tuberculin skin reaction*
Splenectomy	18 ++, 18 ++
Sham splenectomy	18 ++, 16 +
Thymectomy	18 +++, 17 +++
Sham thymectomy	17 +++

* Diameter in millimeters and degree of induration (0 to +++). Each value represents a single animal.

Thymus, spleen, and lymph nodes (brachial, axillary, inguinal, cervical, and mesenteric) were removed from similar donors, placed in Petri dishes containing HBSS, trimmed of fat, and pressed through stainless steel mesh to obtain cell suspensions. These were centrifuged once, resuspended, counted, and injected intravenously in doses of 3–4 × 10^8 into separate recipient rats on the day of irradiation.

Passive Transfer of Tuberculin Hypersensitivity with Lymph Node Cells.—10 to 12-wk-old rats were sensitized by injection of 300 μg of heat-killed tubercle bacilli suspended in oil (Bayol F, Humble Oil & Refining Co., Houston, Tex.) (0.1 ml.) into one hind footpad. 9 days after sensitization the donors were killed and their brachial, axillary, inguinal, cervical, and mesenteric lymph nodes removed. Cell suspensions were prepared in the manner described above, and 4–6 × 10^8 viable nucleated cells were injected intravenously into recipients. The time of transfer following irradiation varied with the experiment.

Skin Testing.—All skin tests were performed by the intradermal injection of 50 μg of tuberculin purified protein derivative (PPD—Parke, Davis & Co., Detroit, Mich.). Reactions were read at 24 hr and scored by measuring their diameter (in millimeters) and degree of induration (0 to +++).

RESULTS

Effect of Splenectomy and Thymectomy on the Delayed Skin Reaction.—Normal rats, 10–12 wk of age, were sensitized by injection of 300 μg of tubercle bacilli suspended in oil into one hind footpad. 10 days afterwards, they were subjected to splenectomy, sham splenectomy, thymectomy, or sham thymectomy, and 5 days later, were skin tested with 50 μg of PPD. Removal of the spleen or the thymus had no effect on the elicitation of intense delayed skin reactions (Table I).

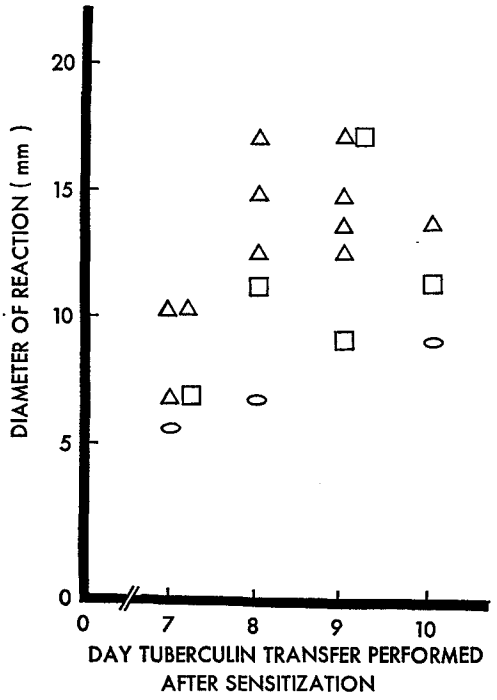

FIG. 1. Effect of time of transfer and dose of transferred lymph node cells on tuberculin sensitivity in recipients. Dose of cells: 2.0–2.5 × 10^8, ellipse; 3.0–3.5 × 10^8, square; and 4.0–6.0 × 10^8, triangle.

Passive Transfer of Tuberculin Hypersensitivity in Normal Rats.—Lymph node cells taken from donor rats 7, 8, 9, or 10 days after sensitization to tuberculin were used for passive transfer to normal recipients. The doses of cells transferred were 2.0–2.5 × 10^8, 3.0–3.5 × 10^8, and 4.0–6.0 × 10^8. Cells taken 8 and 9 day after sensitization gave good delayed reactions in the recipients (Fig. 1), the higher doses in general giving the better responses. The dose of 4.0–6.0 × 10^8 lymph node cells taken at 9 days was chosen for subsequent studies.

Passive Transfer in Thymectomized, Irradiated Rats Restored with Normal Bone Marrow.—The scheme shown in Fig. 2 depicts the general precodure used in our passive transfer experiments:

"Blank slate" recipients were produced by subjecting rats to thymectomy and 900 R of total-body X-irradiation. On the day of irradiation, these animals were infused with varying doses of normal, syngeneic bone marrow; some received no marrow. At different times after irradiation and bone marrow infusion, each rat received a passive transfer of tuberculin sensitized lymph node cells and was skin tested 24 hr later. An aliquot of each pool of lymph node

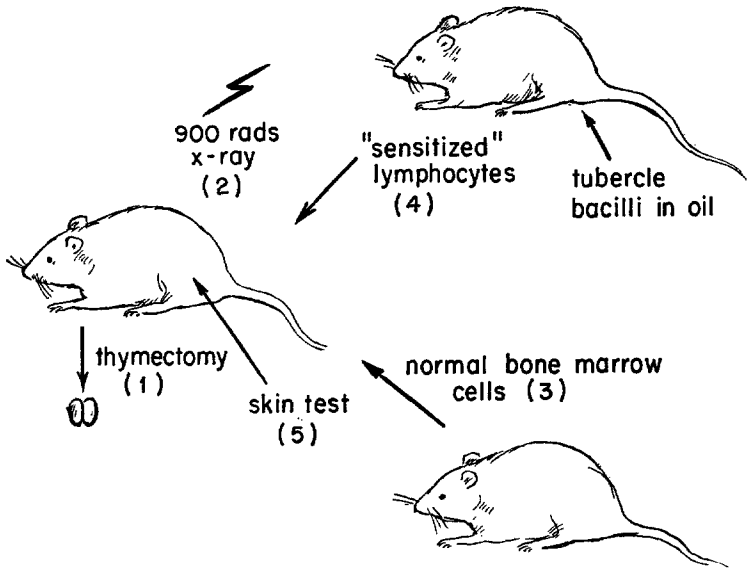

FIG. 2. Schematic depiction of passive transfer experiments.

cells was also injected into a normal recipient as a control for the effectiveness of transfer.

Both the macroscopic and microscopic appearances of the 24 hr skin reactions in the recipient rats were those of classical tuberculin reactions. Fig. 5 shows one such lesion in a rat which had received 2.0×10^8 normal bone marrow cells followed by sensitized lymphocytes on day 10. This reaction measured 16 mm in diameter and had two plus (++) induration. There was no necrosis accompanying the infiltration. Fig. 6 shows, histologically, the lesion with characteristic infiltration of lymphocytes and histiocytes, both perivascular and perifollicular as well as diffuse throughout the dermis.

The results of quantitative experiments are shown in Table II and Fig. 3. Rats receiving a passive transfer on the day of irradiation (0 days), but not

restored with bone marrow, gave insignificant delayed reactions. A skin reaction of 6 mm or less was considered negative since unsensitized rats, whether irradiated or unirradiated, gave average reactions measuring 6 mm in diameter. When low doses of marrow cells were injected into the thymectomized, irradiated recipients, even moderate reactions were not obtained until these cells

TABLE II

Relationship between Dose of Bone Marrow, Interval before Transfer, and Skin Reactivity in Recipients

No. of bone marrow cells $\times 10^8$	Diameter of skin reactions* after sensitized cell transfer on day:				Control‡
	0	5	7	10	
	mm	*mm*	*mm*	*mm*	
0.0	6.3 ± 3.7	—	—	—	**14.1 ± 2.3**
0.125	—	6.7 ± .05	8.3 ± 3.4	**11.7 ± 1.3**	**14.3 ± 1.4**
0.5	—	5.8 ± 1.8	**10.5 ± 2.8**	**13.3 ± 3.6**	**13.6 ± 1.8**
2.0	9.5 ± 2.2	9.6 ± 3.8	**10.5 ± 3.4**	**13.8 ± 3.9**	**13.4 ± 2.2**
2.5–3.0	9.6 ± 1.6	**10.0 ± 2.8**	**13.4 ± 3.5**	**13.4 ± 2.7**	**13.5 ± 2.8**
3.5–4.0	**11.0 ± 2.0**§	**11.3 ± 2.7**	**14.8 ± 2.4**	**15.3 ± 2.3**	**12.4 ± 2.2**

* Skin test performed 24 hr after passive transfer and read 24 hr later. Values given represent averages obtained in groups of 4–10 rats ± standard deviation.

‡ Normal recipients unirradiated and given no bone marrow. A separate control group was set up with each experimental group, but test results were quite uniform and are presented here in a single average figure.

§ Values in boldface type represent reactions greater than 10 mm.

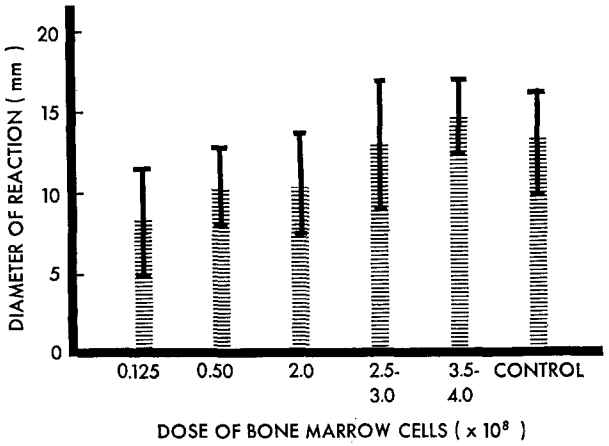

FIG. 3. Average diameter of 24 hr skin reactions of thymectomized, irradiated rats given various doses of normal syngeneic bone marrow and sensitized lymph node cells 7 days later. Each group contained 4–10 rats. I bar represents two standard deviations.

were in residence for 7–10 days before transfer. With high doses of marrow, a shorter period was adequate to permit intense reactions. With the highest dose of 3.5–4.0 $\times$ 10^8 cells, substantial reactions occurred in rats even when the lymph node cells were given on the day of irradiation and bone marrow infusion. Fig. 3 shows clearly the progressive increase in reaction size with increasing doses of bone marrow.

When nonthymectomized rats were irradiated, given 3.0–4.0 $\times$ 10^8 normal bone marrow cells, and sensitized lymphocytes all on the same day, their average PPD reactions at 24 hr were indistinguishable from those in thymectomized animals (Fig. 4).

Passive Transfer in Thymectomized, Irradiated Rats Restored with Normal

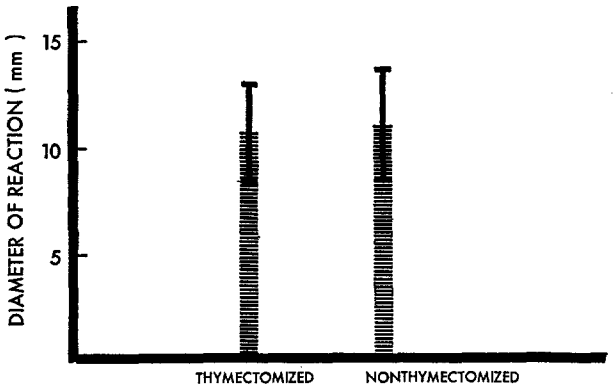

FIG. 4. Effect of presence of the thymus on 24 hr skin reactions of irradiated rats given 4.0 $\times$ 10^8 normal syngeneic bone marrow and sensitized lymph node cells on the same day. Each group contained five rats.

Cells from Sources Other Than Bone Marrow.—In control experiments, 3.0–4.0 $\times$ 10^8 normal spleen, lymph node, thymus, or peritoneal exudate cells were injected *in lieu* of normal bone marrow cells into thymectomized, irradiated recipient rats. Each received sensitized lymph node cells on the same day and was skin tested 24 hr afterwards. None of the reactions obtained with these other cell types approached the value obtained in animals receiving bone marrow cells (Table III). With three of the four tissues, the reaction sizes were hardly greater than those of rats that received no normal cells. In the fourth tissue, the lymph nodes gave an average reaction of intermediate intensity.

DISCUSSION

The data presented in this paper show that normal bone marrow cells are necessary for the production of intense tuberculin reactions in the passive transfer situation. These data strongly support the finding (14–16) that bone marrow

is the actual source of the majority of cells in passively transferred skin reactions of delayed hypersensitivity, cells described as histiocytes or macrophages. It appears that rather high numbers of bone marrow cells are required to permit the elicitation of skin reactions in recipient rats. These may be provided either by infusing the animals with an intial high dose of cells, at the time of sensitized node cell transfer, or by giving a low dose of marrow and allowing sufficient time to elapse before the passive transfer of tuberculin sensitivity. A period of 7–10 days appears to permit sufficient cell multiplication to provide a threshold level of reactive marrow cells.

Equal numbers of cells collected from tissues other than the bone marrow were found not to support elicitation of moderate or intense tuberculin reactions in recipients of sensitized lymph node cells. Interestingly, peritoneal cells, which include a high percentage of macrophages also derived from the bone

TABLE III

Average Diameter of Skin Reactions (mm) after Sensitized Cell Transfer in Rats Injected with Cells from Various Normal Tissues

Source of cells	24 hr tuberculin skin reaction
Bone marrow	11.0
Peritoneal exudate	7.6
Spleen	7.3
Lymph node	8.6
Thymus	7.6
No cells	6.3

Each group contained four rats.

marrow (17), could not be substituted for the marrow cells in this experiment and apparently do not serve as precursors of cells accumulating at the skin test sites. It seems probable, therefore, that the marrow-derived cells in the reactions can only be derived from dividing precursor cells early in their maturation process and that, once the cells become differentiated to mature macrophages, they can no longer circulate or perhaps can no longer leave the blood stream to participate in inflammatory reactions.

The fact that tuberculin reactions of intermediate size were obtained when normal lymph node cells were used raises an interesting point. The precursors found in bone marrow must also be present for a time in the circulation, and they may recirculate in small numbers through the lymph nodes. Micklem et al. (18) have shown that bone marrow cells migrate to and proliferate in the nodes of mice. The possibility that precursors originating in the lymph nodes travel to the marrow and then to the skin reaction appears to be ruled out by the observation that lymph node cells do not migrate to bone marrow (18).

It is probable that bone marrow cells go directly to the skin site without the need to travel through other organs. Removal of the spleen or thymus 5 days before skin-testing actively sensitized rats did not affect their reactivity. Similarly the absence of the thymus in irradiated recipients did not lessen their ability to react; this finding provides additional evidence that the macrophage precursors do not sojourn within this organ when going from marrow to reaction site.

One incidental observation deserves comment. Recipients irradiated and restored with the highest dose of normal bone marrow cells, upon subsequent passive transfer of tuberculin sensitivity, gave skin reactions somewhat larger than unirradiated recipients given similar numbers of cells from the same pool of sensitized lymphocytes (Fig. 3). Rats in the former group possess a much diminished peripheral lymphoid cell compartment as compared to the latter. Therefore the transferred sensitized cells must constitute a higher percentage of the total peripheral lymphocyte population in these recipients. This difference may result in greater expression of the sensitized cells and be responsible for the larger delayed skin reactions actually seen.

SUMMARY

Splenectomy or thymectomy of adult Lewis rats following sensitization with tubercle bacilli did not affect their ability to develop delayed skin lesions upon skin testing with PPD. The presence of the thymus also had no significant effect on reactivity of the recipients in passive transfer experiments. The passive transfer of tuberculin hypersensitivity with sensitized lymph node cells to thymectomized, irradiated recipients depended on the simultaneous or prior injection of normal bone marrow cells. When lymph node cell transfer was performed shortly after irradiation and injection of marrow, high doses of marrow cells (3.5–4.0 $\times 10^8$) were required to permit eliciting reactions of reasonable intensity. If, however, periods of 7–10 days elapsed between the injection of bone marrow and sensitized lymph node cells, lower doses of marrow were sufficient for comparable reactions. Normal thymus, spleen, lymph node, or peritoneal exudate cells, even at high doses could not be substituted for the bone marrow in producing good tuberculin reactions.

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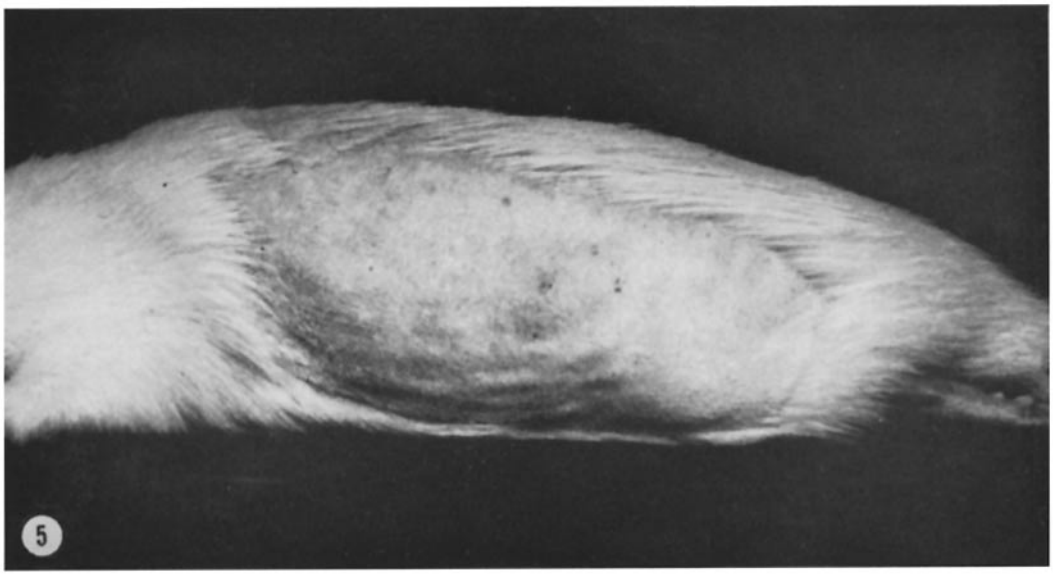
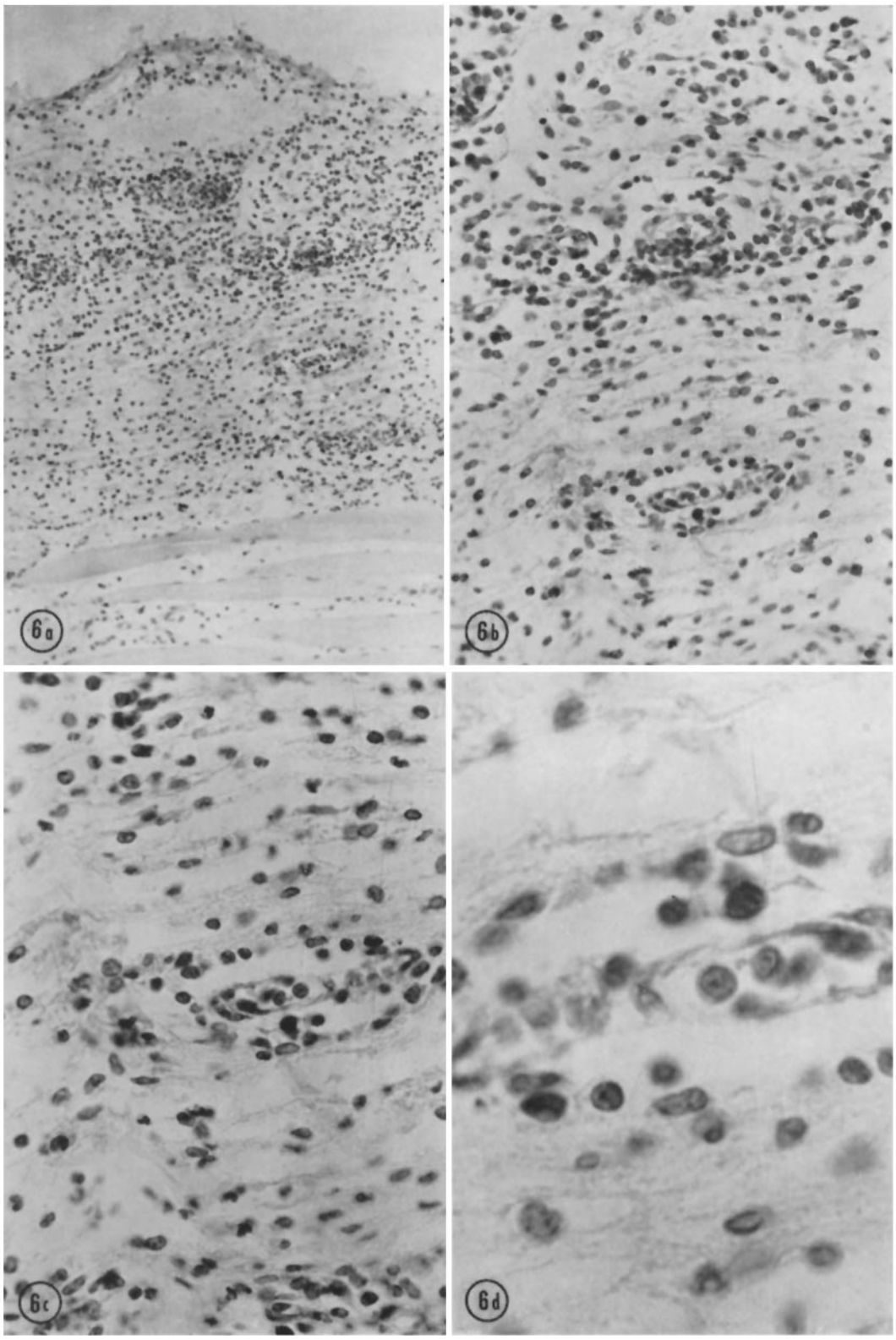


FIG. 5. Tuberculin reaction in rat injected with 2.0×10^8 normal syngeneic bone marrow cells and sensitized lymphocytes 10 days later. Reaction measured 16 mm in diameter and had +++ induration.

FIG. 6. Section of tuberculin reaction in thymectomized, irradiated rat injected with normal bone marrow cells and sensitized lymphocytes. Hematoxylin and eosin. *a.* ×35; *b.* ×100; *c.* ×250; *d.* ×970.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