BONE MARROW AS SOURCE OF CELLS IN REACTIONS OF CELLULAR HYPERSENSITIVITY

II. Identification of Allogeneic or Hybrid Cells by Immunofluorescence in Passively Transferred Tuberculin Reactions*‡

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In previous papers (1, 2), we have shown that bone marrow cells are essential for the production of tuberculin skin reactions and that these cells may be found in great numbers at the specific reaction sites. In the present publication the cell populations in skin reactions of tuberculin hypersensitivity are analyzed by immunofluorescence.

Materials and Methods

Animals.—Male and female rats of the inbred Lewis, BN, and DA strains and F_1 hybrids of (BN × L) and (DAxL) crosses were used. The Lewis and BN rats were obtained from Microbiological Associates, Inc., Bethesda, Md. The DA rats were kindly supplied by Dr. Darcy B. Wilson, Department of Medical Genetics, Philadelphia, Pa. F_1 hybrids were bred in our own animal colony.

Preparation of Recipients.—Male Lewis rats used as recipients were thymectomized at 5–7 wk (2, 3) and given 900 rads of total body X-irradiation at 8–10 wk of age under the conditions outlined in reference 2. They received a single intravenous dose of 4.0×10^8 nucleated bone marrow cells from normal donors, 3–4 months of age, on the day of irradiation (2).

Passive Transfer of Tuberculin Hypersensitivity with Lymph Node Cells.—Techniques of sensitization, cell preparation, cell transfer, and skin testing are described in the previous paper (2). Some rats were skin tested with 20% turpentine (high grade artist's turpentine) in olive oil (Shurfine imported olive oil) as a nonspecific control.

Fluorescence Assay.—The technique originally described by Möller (4) for the detection of mouse H-2 isoantigens was adapted for the present study.

Preparation of Cells for Study.—Cells were studied from the following sites: skin test reactions (tuberculin and turpentine), bone marrow, spleen, mesenteric, and axillary lymph node. Suspensions were prepared from specific and nonspecific skin reactions by teasing the underside of the excised reaction sites, from bone marrow by repeated washings of the femurs, and

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from spleen and lymph nodes by pressing through stainless steel gauze. The cells were centrifuged at 4°C at 180 *g* for 10 min and resuspended in Hanks' balanced salt solution (HBSS—from Grand Island Biological Company, Grand Island, N. Y.) at an approximate concentration of 1×10^7/ml. Typical cells teased from 24 hr tuberculin reactions are shown in Fig. 2. A few were lymphocytic but most had the morphologic character of monocytes or histiocytes. These resembled closely the cells seen in histologic sections. Polymorphonuclears never exceeded 1% of the cell population.

Preparation of Isoantisera.—Isoantisera were produced in rats by three intraperitoneal injections of aqueous suspensions of $2.5\text{–}5.0 \times 10^8$ homologous lymphoid cells (pooled spleen and lymph nodes), at 2 wk intervals. 6 days after the last injection the animals were bled by cardiac puncture. The sera were pooled and heat inactivated at 56°C for 30 min.

Fluorescein Conjugate.—A commercial preparation of fluorescein conjugated rabbit anti-rat globulin (Microbiological Associates, Inc., Bethesda, Md.) was used, undiluted, and unabsorbed. There was no nonspecific staining when living cells were studied (4).

TABLE I

The Detection of BN Antigen by Immunofluorescence in Mixtures of BN and Lewis Cells

Per cent BN cells added	Per cent Lewis cells added	Per cent stained	Per cent unstained
100	0	89.4	10.6
75	25	61.7	38.3
50	50	44.6	55.4
25	75	21.5	78.5
0	100	7.15*	92.8

* Injury reaction: entire cell stained.

In these experiments, approximately 200 cells were counted in each mixture.

Staining Techniques.—The indirect or "sandwich" method was used throughout. A 1 ml aliquot of each sample, containing 1×10^7 cells/ml, was centrifuged at 180 *g* for 10 min in 10 × 75 mm tubes. The supernatant was discarded and replaced by 0.5 ml of antiserum, in dilutions varying from 1:4 to 1:6 in different experiments. The cells were resuspended by shaking and incubated at room temperature for 15 min. After centrifugation and two washes with 1.0 ml volumes of HBSS, the cells were incubated in 0.1 ml of the conjugated rabbit anti-rat globulin for another 15 min at room temperature. After two more washings, the cells were resuspended in 0.2 ml to 0.5 ml of HBSS. The tubes were placed in an ice bath until study. The cells were studied on the day of staining, usually within 30 min after completion of the staining procedure.

Microscopy.—A drop of each stained cell suspension was placed on a chemically clean microscope slide immediately prior to examination and covered with a cover glass. The preparations were examined with a Zeiss photomicroscope fitted with two light sources, a tungsten lamp and a Sylvania HGK-200 ultraviolet lamp. A 3 mm thick BG-12 exciter filter and numbers 53 and 47 barrier filters were used.

In any one field of view, the total number of nucleated cells was counted under darkfield illumination. The light source was then switched to UV and the total number of fluorescent cells counted. Typical fields are shown in Fig. 2*c* and *d*. For most samples, over one hundred cells were counted and the *per cent* fluorescent cells calculated.

RESULTS

Specificity of the Immunofluorescence Method.—Lewis anti-BN and Lewis anti-DA sera were tested in the fluorescence assay against BN or DA target cells. Cells obtained from lymph nodes, bone marrow, and tuberculin skin reactions all exhibited bright fluorescence with serum dilutions between 1:2 and 1:6. Normal Lewis serum never produced any fluorescence. Cells of (BN × L)F_1 and (DA × L)F_1 hybrids also were stained strongly with the appropriate antisera.

To test the specificity of the fluorescence, lymphoid cells from BN and Lewis rats were mixed in varying proportions and assayed with undiluted Lewis anti-BN antiserum. With each mixture, the percentage of stained cells actually observed was very close to the proportion of BN cells added (Table I). However, 10–15% of these failed to stain. Cytoplasmic uptake of fluorescein label by some of the cells in the suspension was attributed to nonspecific "injury reaction." Such cells were excluded from the calculations presented below.

TABLE II

Distribution of Cells from Transfused Hybrid Bone Marrow in Tissues of Recipient Rats

Time after bone marrow infusion	Fluorescent cells, %			
	Bone marrow	Spleen	Mesenteric lymph node	Axillary lymph node
Same day	40.5	53.8	33.2	6.9
7 days	94.9	93.6	61.2	35.3

Each figure represents the average of values obtained in three rats.

Repopulation of Lymphoid Tissues in Thymectomized, Irradiated Rats Restored with Hybrid Bone Marrow.—The tissues of thymectomized, irradiated Lewis rats restored with hybrid marrow cells immediately after irradiation were studied on day 0 and day 7 (Table II). 1 hr after bone marrow infusion, the tissues still included appreciable numbers of host cells; the numbers of donor cells varied from about one-half in the marrow and spleen to less than 10% in the axillary lymph node. On day 7, however, essentially all of the cells in the marrow and spleen and one- to two-thirds of those in the lymph nodes were donor cells.

Immunofluorescence Assay of the Cellular Infiltrate in Tuberculin Skin Reactions.—In thymectomized, irradiated Lewis rats injected with normal BN or DA bone marrow, and given sensitized Lewis lymphocytes 7 days later, skin reactions to PPD showed considerable variability in size (1). Occasional animals treated in this manner developed homologous disease. These immuno-

fluorescence data appeared to demonstrate, nevertheless, that the majority of cells in tuberculin reactions were derived from the injected bone marrow (1).

In further experiments, F_1 hybrid rats of (BN × L) and (DA × L) crosses were used as marrow donors, and more consistent results were obtained (Table III). Passive tuberculin reactions in these recipients contained 66% to 88% bone marrow-derived cells. A comparable proportion of marrow-derived cells

TABLE III

*Origin of Infiltrating Cells in Specific and Nonspecific Skin Reactions Elicited in Rats Given Hybrid Bone Marrow**

Strain of donor marrow	Tuberculin reaction	Fluorescent cells, %					
		Reaction sites		Lymphoid tissues			
		PPD	Turpentine	Bone marrow	Spleen	Mesenteric lymph node	Axillary lymph node
	mm						
(DA × L)	15 ++‡	79.0	—	98.1	38.1	16.0	14.0
(DA × L)	17 ++	66.0	86.7	95.0	33.6	14.3	10.7
(BN × L)	11 ++	86.0	86.5	97.5	25.7	27.0	—
(DA × L)	17 +±	75.5	84.2	94.3	29.3	25.5	28.2
(BN × L)	16 ++	88.0	87.3	97.3	71.5	31.6	39.6
(BN × L)	14 ++	78.0	—	88.0	29.3	19.6	18.0
Mean	15.0	78.8	86.2	95.0	37.9	22.3	22.1
S.D.	±2.8	±7.2	±1.2	±3.6	±15.5	±6.2	±10.5

* Passive transfer of tuberculin sensitivity performed 7 days after irradiation and bone marrow infusion.

‡ Degree of induration measured subjectively on scale of 0 to +++.

A portion of these data were published earlier (see reference 1).

was found among the small number of cells teased from nonspecific inflammatory reactions induced by turpentine, in confirmation of the findings of Volkman and Gowans (5). The bone marrow of the recipients was almost entirely made up of cells derived from the injected marrow, while the other lymphoid organs contained much fewer of these.

Since intense delayed skin reactions could be obtained in rats given both normal bone marrow and sensitized lymph node cells on the same day (2), an immunofluorescence assay was carried out with the rats treated in this manner, again with F_1 hybrid rats as bone marrow donors (Table IV). Again marrow-derived cells constituted the great majority of the infiltrating cells in the tuberculin reactions (66.6% to 84.5%). High percentages were also found in the turpentine reactions and in the marrow of the recipients. The spleen and lymph

TABLE IV

*Origin of Infiltrating Cells in Specific and Nonspecific Skin Reactions Elicited in Rats Given Hybrid Bone Marrow**

Strain donor marrow	Tuberculin reaction	Fluorescent cells, %					
		Reaction sites		Lymphoid tissues			
		PPD	Turpentine	Bone marrow	Spleen	Mesenteric lymph node	Axillary lymph node
	mm						
(DA × L)	17 ++‡	74.5	74.0	—	24.0	6.7	14.3
(DA × L)	18 ++	82.8	—	—	22.1	6.6	16.8
(DA × L)	18 ++	67.5	66.0	84.5	23.8	7.0	20.6
(DA × L)	17 +++	66.6	60.5	81.5	27.6	12.9	9.5
(DA × L)	15 +	84.5	70.7	88.0	13.5	3.7	—
Mean	17.0	75.2	67.8	84.7	22.2	7.4	15.3
S.D.	±1.1	±7.5	±5.7	±2.7	±4.7	±3.1	±4.4

* Passive transfer of tuberculin sensitivity performed on day of irradiation and bone marrow infusion.

‡ Degree of induration, measured subjectively on scale of 0 to +++.

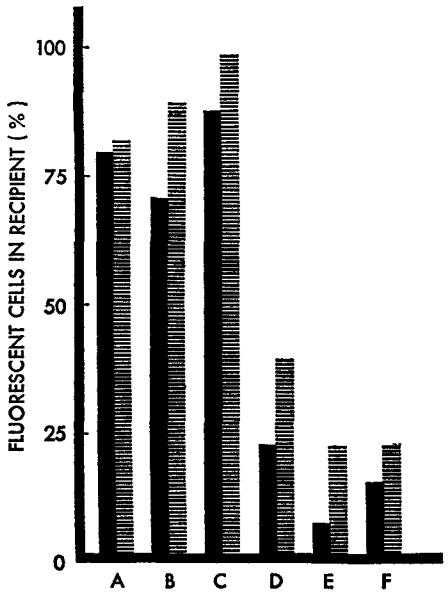

FIG. 1. Per cent fluorescent cells in tissues of recipient rats. Lymphocytes given either same day or 7 days later. Each group contained five to six rats. Same day, solid column; 7 days, striped column. A. tuberculin reaction; B. turpentine reaction; C. bone marrow; D. spleen; E. mesenteric lymph node; F. axillary lymph node.

TABLE V

Origin of Infiltrating Cells in Tuberculin Skin Reactions Elicited in Rats Given Syngeneic Bone Marrow and Sensitized Hybrid Lymph Node Cells

Reactions studied 9 hr after skin test

Strain donor lymphocytes	Tuberculin reaction	Fluorescent cells (%)				
		Reaction Site	Lymphoid tisssue			
		PPD	Bone marrow	Spleen	Mesenteric	Axillary
	mm					
(DA × L)	11 +*	38.0	9.6	71.5	86.0	88.5
(BN × L)	8 ±	29.2	8.0	88.7	93.0	85.2
(DA × L)	11 ±	34.0	14.7	85.2	89.5	—
(DA × L)	9 +	30.5	1.9	90.3	79.2	—
Mean	9.7	32.9	8.6	83.9	86.9	86.9
S.D.		±2.9	±4.5	±7.4	±5.2	±1.7
Reactions studied 16 hr after skin test						
(DA × L)	10 +	39.3	23.1	74.8	98.4	90.2
(DA × L)	10 ±	27.3	19.2	87.0	97.5	84.0
(BN × L)	15 ++	31.7	12.5	79.5	86.7	81.0
Mean	11.7	32.8	18.3	80.4	94.2	85.1
S.D.		±4.9	±4.4	±5.1	±5.3	±3.8
Reactions studied 24 hr after skin test						
(DA × L)	13 +	40.3	21.2	86.3	93.0	91.3
(DA × L)	9 ±	16.0	9.6	84.0	86.7	84.0
(DA × L)	11 +	20.6	6.7	88.7	94.4	82.4
Mean	11	25.6	12.5	86.3	91.4	85.9
S.D.		±10.5	±6.3	±1.9	±3.4	±3.9

* Degree of induration, measured subjectively on scale of 0 to +++.

nodes possessed very few fluorescent cells. The results when the passive transfer was performed on the day of irradiation and bone marrow infusion and on the 7th day were closely similar (Fig. 1). The values were slightly higher in the 7 day experiments perhaps corresponding to the relative increase of donor marrow cells seen in the marrow and other lymphoid organs (Table II).

Finally thymectomized, irradiated Lewis rats were infused with normal Lewis

bone marrow cells and sensitized F_1 hybrid lymph node cells on the same day, and were skin tested the next day with PPD. The reactions were read and the cells examined by immunofluorescence 9, 16, and 24 hr after skin test (Table V). Lymph node–derived cells were found to make up one-quarter to one-third the cells in the tuberculin reactions at the three intervals studied. There appeared to be no change in the proportion of different cell types arriving at delayed skin reaction sites as the lesions developed. As might be expected the bone marrow contained very few of the hybrid lymphocytes while the peripheral lymphoid organs had very high numbers. Essentially all the cells in all tissues studied appeared to be derived from either the transfused marrow or the transferred sensitized lymph node cells (Table VI). The host in these experiments contributed little or not at all either to the reactions observed or to

TABLE VI

Total Cell Populations in 24 hr Tuberculin Skin Reactions

Bone marrow donor	Lymph node donor	Fluorescent cells (%)				
		Reaction site	Lymphoid tissues			
		PPD	Bone marrow	Spleen	Mesenteric	Axillary
Hybrid	Syngeneic	75.2	84.7	22.2	7.4	15.3
Syngeneic	Hybrid	25.6	12.5	86.3	91.4	85.9
Total		100.8	97.2	108.5	98.8	101.2

the repopulation of lymphoid organs. The transferred cells showed a high degree of specificity, marrow going to marrow, and peripheral cells to spleen and lymph nodes.

DISCUSSION

The experiments reported here clearly demonstrate that cells derived from the bone marrow constitute the great majority of the cellular infiltrate in tuberculin reactions in the rat. Contact reactions in the guinea pig have similarly been shown, by the use of ^{3}H-thymidine labeling, to contain a preponderance of marrow-derived cells (6). The percentage of such cells in our experiments were approximately the same whether lymphocyte and bone marrow transfers were performed on the same day or when a period of 7 days elapsed between the two transfers. However, since the experiments were carried out in a relatively artificial system, i.e. thymectomized, irradiated, animals restored with arbitrary numbers of marrow cells and "sensitized" lymph node cells, these percentages may not correspond to the proportions of such cells participating in lesion formation in actively sensitized animals. Of the lymph node cells transferred, only a

small fraction can be presumed to have been specifically sensitized cells and the proportion of sensitized cells at the reaction sites must therefore have been considerably less than the 25 odd *per cent* found by immunofluorescence. In studies in which ^{3}H-thymidine labeling was used (7–10), specific cells appeared to make up less than 10% of the total infiltrating cells. In actively sensitized animals given ^{3}H-thymidine, hematogenous histiocytic cells derived from actively dividing precursor cells—presumably these correspond to the marrow-derived cells studied here—made up the majority of cells at all stages in the evolution of the lesion (11, 12).

Histologic studies (13) and studies with tritiated thymidine suggest that a closely similar mechanism must be involved in tuberculin and similar reactions (11), contact allergy (8), autoallergic lesions such as encephalomyelitis (12) and thyroiditis (14), skin homograft rejection (15), and the disseminated lesions of adjuvant disease (16). The inference from the present work and that recently published by Liden (6) is that the inflammatory cells in many and perhaps all of these will prove to have the same bone marrow origin. This inference is supported, in the case of adjuvant disease, by a recent study with the use of the immunofluorescence technique developed in the present investigation[1] and, in the liver lesions of graft-versus-host disease, by a study with the use of the T^6 chromosomal marker (17).

Another inference from the present findings is that the histiocytes or macrophages which constitute the principal infiltrating cells in various delayed reactions (13) are actually the same as reticuloendothelial cells found normally in various parts of the body (18). These travel in the blood stream as monocytes (19) and respond to trauma or nonspecific irritation in the skin (5) or elsewhere (20, 21). They normally have a rapid turnover (22), which accounts for the high degree of labeling in experiments with tritiated thymidine (7–10). The same cells act as the source of macrophages in peritoneal exudates (20). This validates the use of peritoneal cells in in vitro experiments directed at analyzing the mechanism of delayed hypersensitivity (23, 24). The relationship between the precursors of these cells in the marrow and the progenitors of erythrocytes, granulocytes, platelets, and the lymphoid cells which migrate to the thymus is a challenging problem remaining to be investigated.

The possibility that monocytes originating in the bone marrow must sojourn in another organ before travelling to specific tuberculin reaction sites appears to be ruled out in our experiments by the fact that a preponderance of marrow-derived cells was found even when transfer of marrow and sensitized cells were performed on the day of irradiation and skin reactions elicited the following day. The thymus has been eliminated as playing any role in the processing of these cells by the fact that all recipient rats were thymectomized prior to irradia-

[1] Putnam, D. A., D. M. Lubaroff, and B. H. Waksman. 1968. Bone marrow as source of cells in reactions of cellular hypersensitivity. III. Adjuvant arthritis in rats. In preparation.

tion and cell transfer. It must not be overlooked, however, that thymus-derived cells may act as precursors of the sensitized lymph node cells used for transfer of sensitivity (25).

The present findings shed no light on the actual mechanism of the delayed reaction. Available evidence suggests that the local accumulation of monocytes (histiocytes, macrophages) is triggered by the local reaction of sensitized lymphocytes in the circulating blood with antigen. This reaction has been shown to result in release of a protein mediator (26) which, on the one hand, renders macrophages sticky (23, 24) and, on the other, damages other cell types such as fibroblasts (27) and perhaps endothelium (28). Stickiness alone may account for the local diapedesis of these cells, or they may simply be responding to tissue injury as they do at nonspecific sites of irritation (5, 20). The very much greater intensity, however, of the specific lesion (13) implies a mechanism beyond that of a simple response to local damage. The coagulation mechanism plays a role, unknown as yet, in this process (29). Our limited data suggest that there is no marked change in the proportions of reacting sensitized cells and monocytes as progressively larger numbers of cells enter the local site in the evolution of the lesion. It is possible that a transfer of specific RNA occurs between sensitized and nonsensitized lymphocytes (30, 31) or between sensitized cells and macrophages (32), and that this process also adds to the local recruitment of cells.

SUMMARY

The precise origin of cells infiltrating tuberculin skin reactions was studied with the technique of immunofluorescence. Thymectomized, irradiated Lewis rats were restored with bone marrow from allogeneic or F_1 donors. They were passively sensitized to tuberculin by a subsequent transfer of Lewis lymph node cells and were given intradermal skin tests with tuberculoprotein. In 24 hr reactions the majority of cells were shown to be derived from the infused marrow. These results were the same regardless whether the lymphocyte transfer was performed on the day of irradiation and marrow injection or 7 days later. The cells in the tuberculin reactions, marrow, spleen, and lymph nodes not derived from the bone marrow were found to originate in the transferred lymph node cells. The relative percentages of marrow-derived and lymph node–derived cells in the tuberculin reactions remained the same during the 9–24 hr period following skin test.

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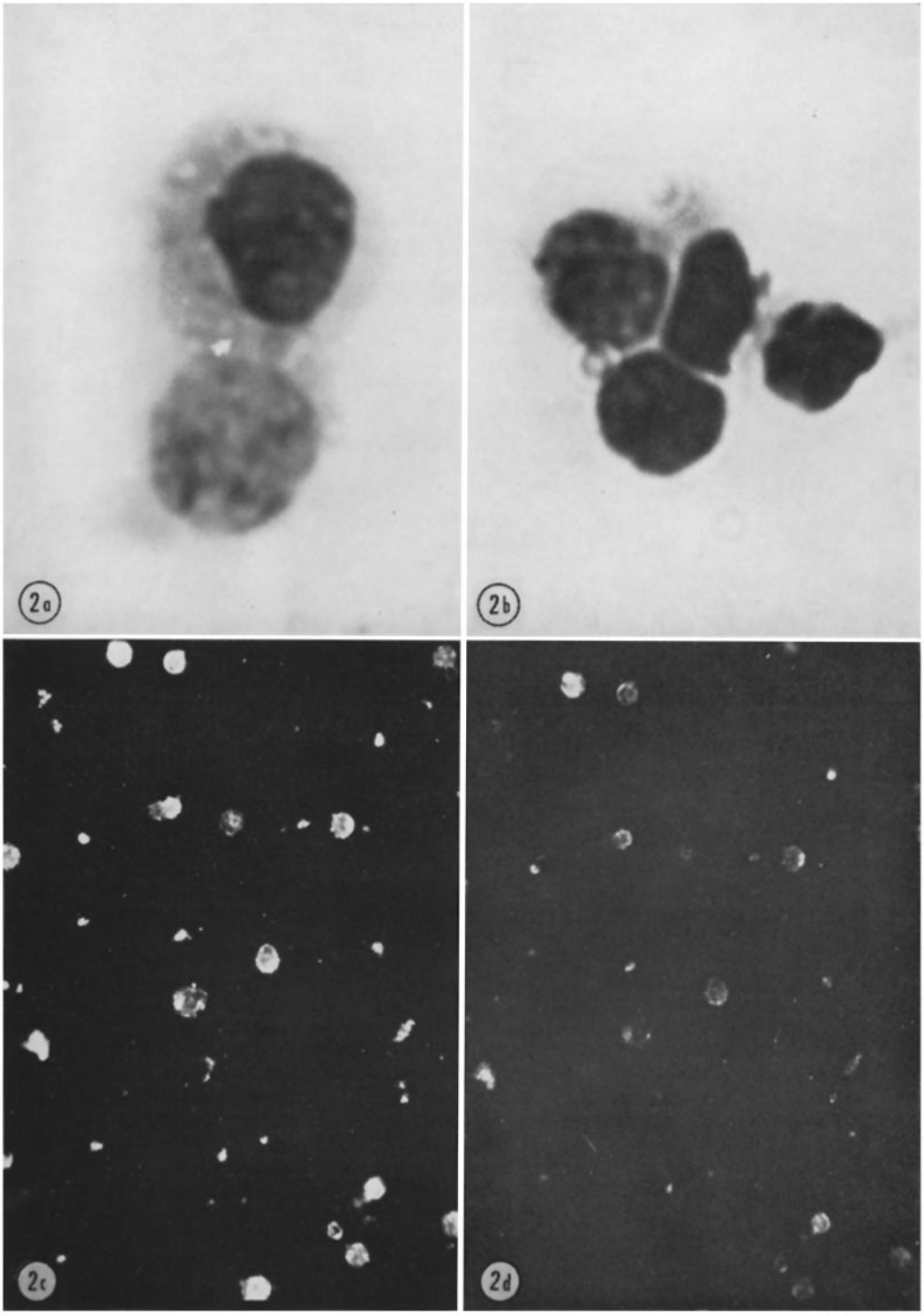
FIG. 2. Examples of cells teased from tuberculin reaction of recipient rat. Giemsa, × 970. *a*. histiocytes; *b*. mixture of lymphocytes and histiocytes; *c*. darkfield photograph of lymphoid cells; *d*. fluorescent photograph of the same cells. Only a portion of the cells show the ring fluorescence. Note the deep staining "injury reaction" in the upper left-hand corner.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