SEPARATION OF A SPLEEN-CELL SUSPENSION BY DENSITY-GRADIENT CENTRIFUGATION AND THE NATURE OF THE IMMUNOLOGICAL FUNCTIONS OF THE INDIVIDUAL CELL FRACTIONS

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Recently conducted experimental studies showed that numerous immunological phenomena—the perception of antigenic irritation, the retention and transfer of immunological information, antibody production, the development of delayed hypersensitivity and transplantation immunity, etc. are functions of the lymphoid tissue. However, lymphoid tissue represents a combination of various cell forms linked by the possibility of various transformations and differentiations. The problem of with which form or with which direction of differentiation this or that immunological phenomenon is linked cannot be considered as resolved at present. One of the ways to resolve it may be the obtaining of cellular elements in the form of uniform suspensions and a subsequent study of the immunological activity of such suspensions. Some authors have successfully obtained uniform suspensions of blood cell elements by density-gradient centrifugation. However, attempts to separate a spleen-cell suspension in this way [14], like the attempt to separate this suspension on a column of small glass beads [12], did not give completely satisfactory results. Holub separated a uniform suspension of small lymphocytes by centrifuging lymph in a density gradient of a sucrose solution [11].

The purpose of the present work was to fractionate a suspension of the spleen cells of immunized animals and to study the relationship between the cellular composition of the obtained fractions and their immunological functions.

EXPERIMENTAL METHOD

A suspension of mouse spleens in Henk's solution was prepared by a method described earlier [5]. This suspension was separated by centrifugation in the periodic density gradient of a sucrose solution. All the solutions of chemically pure sucrose were prepared in Henk's solution with the addition of 20% normal bovine serum without preservatives. Two types of gradients each of which consisted of three layers: the first of 40, 30 and 20% sucrose solutions, the second—of 25, 20 and 15% solution were used in the work. Special experiments showed that conduction across such a gradient does not cause essential changes in the immunological activity of spleen cells. The gradient was prepared in a glass centrifuge cup (inner diameter 45 mm). The volume of each layer was 15-20 ml.

Seven ml of the spleen-cell suspension, containing $2 \cdot 10^8$ nucleated cells in 1 ml, was layered on the surface of the first gradient and carefully centrifuged for 7-8 min at 50-100 g. After this, 7 layers of cells could be differentiated in the cup. Each layer was suctioned off, placed in a separate tube and the sucrose removed by centrifugation in Henk's solution. In each of the suspensions obtained the cell concentration (in a counting cell) and composition (differentiated by counting 1000 cells in smears stained with azure blue-eosin) was determined. The classification of lymphoid tissue cells suggested by M. P. Pokrovskaya et al. [6] was used in the calculation.

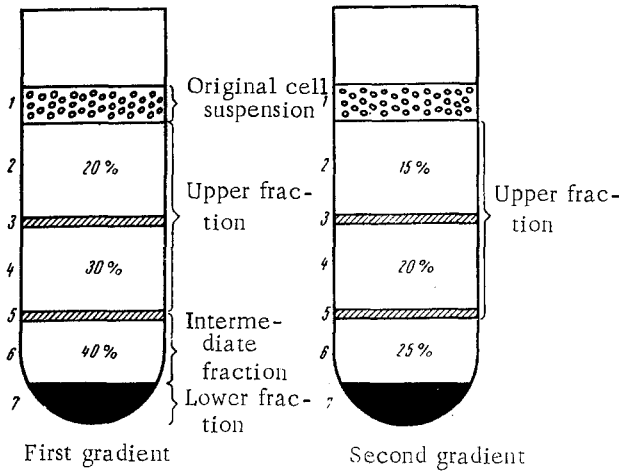

Fig. 1. Sepration of spleen-cell suspensions into periodic density gradients of a sucrose solution. 1-7) Layers of cells after centrifugation (see text).

TABLE 1. Cellular Composition of Fraction of a Spleen Suspension of Mice Immunized with Vi-Antigen (in %)

Cell form	Original suspension	Upper fraction after first gradient	Upper fraction after second gradient	Lower fraction
Myeloid elements . . .	11.5	2.79	0.7	18.4
Erythroid elements . . .	13.1	0.3	0.1	29.5
Blasts	0.6	0.03	0	1.0
Plasmoblasts	0.2	0	0	0.9
Immature plasmatic cells	0.35	0.01	0	1.6
Mature plasmatic cells .	0.45	0.07	0.03	1.9
All cells of plasmatic series	1.0	0.08	0.03	4.4
Lymphocytes	71.5	96.6	99.1	42.7
Other cells	2.3	0.2	0.1	3.9

EXPERIMENTAL RESULTS

A study of the cellular composition of the layers of the first gradient (see Fig. 1) showed that whole cells do not remain in layer after centrifugation. Layers 2, 3, and 4 are identical in cell composition; therefore the material forming these layers were used in the experiments as a single "upper fraction." Sediment at the bottom of the cup formed the "lower fraction" (layer 7). Layers 5 and 6 represented the intermediate fraction and were not used in the experiments.

The cellular compositon of the fractions of a spleen suspension from mice immunized with a preparation of S. typhi Vi-antigen (average data of seven experiments) is presented in Table 1. In the original suspension along with lymphocytes and prolymphocytes, comprising 71.5% of the cells, a considerable contamination by myeloid and erythroid elements is present and a few ferm cells (blasts), on the average 1%, makeup the cells of the plasmatic series. The upper fraction is more uniform in composition than the original suspension, and on the average consists of 96.6% lymphocytes. The most significant contaminants are the myeloid elements. Blasts and cells of the plasmatic series are encountered very seldom. However, the composition of the upper fraction after the first gradient varied in certain experiments while the relative number of lymphocytes varied from 89 to 99%. To obtain a purer suspension of lymphocytes the material of the upper fraction was layered on the second gradient and the whole procedure of centrifugation and separation of layers was repeated. Layers 2, 3, and 4 formed the upper fraction (see Fig. 1). The lower fraction of the second gradient was not used in the experiments.

After passing through the second gradient the upper fraction becomes very uniform in composition and consists of 99.1% lymphocytes. This value hardly changes in individual experiments. Myeloid and erythroid cells are a negligible contaminant. Single mature plasmatic cells (in 10,000 lymphocytes) are not found in any of the experiments. Blasts, plasmoblasts and immature plasmatic cells are usually absent from this fraction. It is possible to consider that the upper fraction of the second gradient represents a practically pure suspension of lymphocytes. The lower fraction, in comparison with the original suspension, is enriched with myeloid and erythroid cells and, mainly, with cells of the plasmatic series (4.4% instead of 1% in the original suspension).

The difference in cellular composition of the spleen suspension fractions is shown in Fig. 2. The difference in the content both of lymphocytes and cells of the plasmatic series among all the fractions studied is statistically significant.

A study of the immunological activity of the fractions was done on two models. The first of them was the production of antibodies by mice immunized with intravenous polysaccharide antigen—S. typhi Vi-antigen [1]. Intensive production of Vi-antibodies in these experiments occurs mainly in the spleen they can be transferred to the intact mouse-recipients by transplantation of a spleen suspension from immunized donors [5]. To resolve the problem

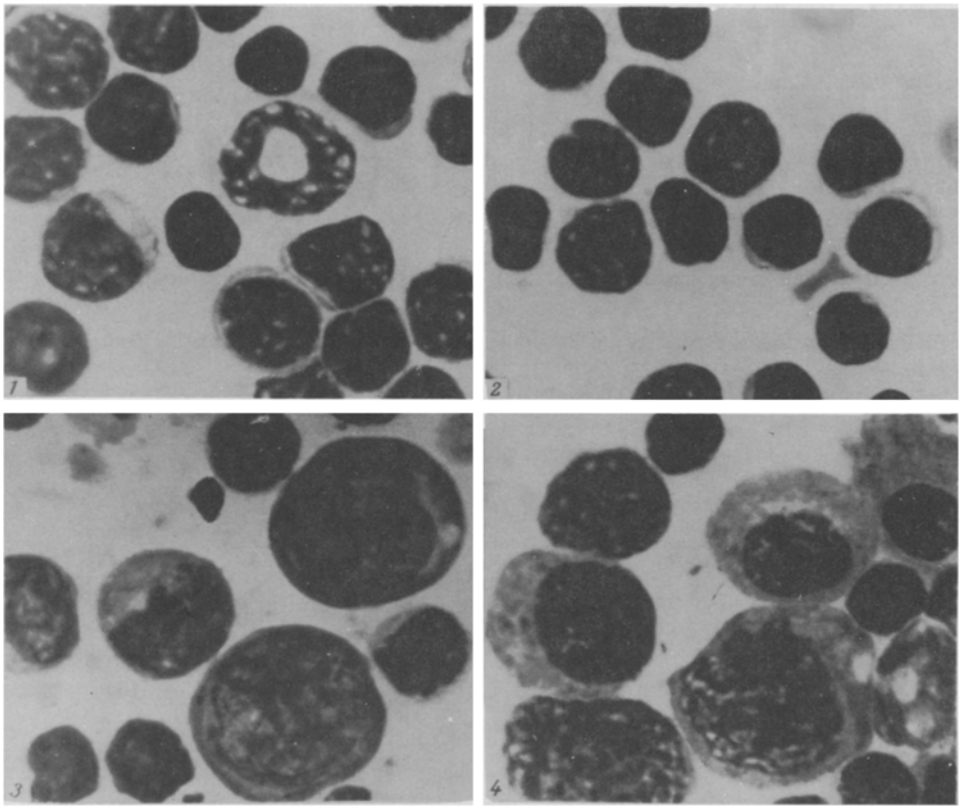

Fig. 2. Cellular elements of various fractions of spleen suspension. 1) Upper fraction after 1st gradient; 2) lower fraction after 2nd gradient consists of uniform lymphocyte suspension; 3, 4) lower fraction after 1st gradient, many blasts, plasmoblasts and plasmatic cells.

of with which fraction of the spleen suspension the production of the Vi-antibodies is connected in the productive phase, five uniform experiments were set up on 148 mice (Table 2). Mouse donors (of the CC57 BR line) were immunized intravenously with 1 μg of Vi-antigen; after three days (on the first day of the productive phase of antibody production) fractions were prepared from their spleens by the above described method which were transplanted to intact recipients of the same line in the amount of 10^8 cells per recipient. One to two days after transplantation blood was taken from the recipients and the Vi-antibodies in the serum determined by the passive hemagglutination reaction [3]. The titer of the blood Vi-antibodies served as an index of the participation of the transplanted fraction in antibody production.

From Table 2 it is seen that the upper fraction did not produce antibodies in the recipient organism in any of the experiments; in other words, the small lymphocytes in the productive phase of antibody formation are not active participants in the synthesis of immune globulins. On the other hand, the cells of the lower fraction produced considerably more antibodies than the cells of the original spleen suspension. This can be explained by the considerably greater number of cells of the plasmatic series in the lower fraction (see Table 1).

The second experimental model was used to study the question of the cellular bases of the response to revaccination. It is shown that a single immunization of mice of the CC57 BR line with complete S. typhi 0-901 antigen does not lead to the production of an observable amount of antibodies, while repeated injection of the same antigen causes rapid and intensive antibody production in the pattern of a response to revaccination. The capacity for a revaccination response may be transferred to the intact mouse recipients by transplanting in them a suspension of spleens of donors immunized once with O-antigen [4].

In the present work the mouse donors were immunized with a single intravenous injection of 1 μg of O-antigen, after 20 days a suspension of their spleens was separated in a sucrose gradient and the fractions obtained transplanted

TABLE 2. Vi-antibody in Recipients of Various Fractions of Spleen Suspension

Transplanted material	Vi-antibody in recipients in experiments					Average titer
	№ 1	№ 2	№ 3	№ 4	№ 5	
Original spleen suspension	—	40	40	160	320	96
Upper fraction		0	0	0	0	0
Lower fraction	320	320	1 280	2 560	2 560	955

Note. All figures in the table are values of the inverse titer of Vi-antibodies.

TABLE 3. Transfer of Capacity for Response to Revaccination by Various Fractions of Spleen Suspension

Donor	Transplanted material	O-antibodies of recipients in exps.							Average titer
		№ 1	№ 2	№ 3	№ 4	№ 5	№ 6	№ 7	
Singly immunized with O-antigen	Original suspension	320	—	—	—	160	40	80	110
	Upper fraction	320	640	160	640	180	80	80	223
	Lower fraction	160	80	320	320	320	80	160	175
Nonimmunized	Original spleen suspension	0	—	—	0	0	—	—	0

Note. All figures are the values of the inverse titer of O-antibodies in recipients of the given suspension (7 days after single injection of O-antigen).

to intact recipients. A day after the transplantation the recipients were injected with O-antigen. The appearance of O-antibodies (determined by the passive hemagglutination reaction) in the recipients' serum served as an indication that the transplanted cellular material possesses an "immunological memory" function and is the morphological substrate of the revaccination response. The results of 7 such experiments (on 228 mice) are presented in Table 3. The capacity for revaccination response is transmitted both by the upper and lower fraction of the spleen suspension. Let us emphasize the fact that a lymphocyte suspension not containing germ cells transmitted this capacity to the recipient.

Consequently, the small lymphocytes possess a clearly expressed ability to retain immunological information. However, it remains unclear whether the lymphocytes are the only carriers of an "immunological memory." The lower fraction also possesses this function, which may be explained both by the presence in it of a large number of lymphocytes and also by the fact that other cellular forms may serve as custodians of immunological information.

The method used for separating a suspension of lymphoid cells by centrifugation in a density gradient of a sucrose suspension may become a valuable auxiliary method in immunological studies. It made it possible to confirm the idea [2, 10, 13, 16] that intensive antibody production is the function of cells of the plasmatic series. It is particularly important that with the help of this method it is possible to isolate a practically pure fraction of small lymphocytes and by this to contribute to the resolution of the problem of the immunological potencies of these cells.

The data presented shows that one of the functions of the lymphocytes is the retention of immunological information, that is, an "immunological memory." On the basis of our previous observations [7-9] and also the data of other authors [15] it may be assumed that lymphocytes not only retain immunological information but also transfer it to various lymphoid organs.

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