CLONES OF ANTIGEN-BINDING CELLS IN HEMATOPOIETIC SPLENIC COLONIES

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Antigen-binding cell clones and Ig-positive cells were found and quantitatively assessed in primary hematopoietic splenic colonies. The results were analyzed on the basis of the hypothesis, the validity of which is discussed, that the ratio of clone volumes of specialized B-cells should reflect the quantitative ratio between the corresponding V-genes in a given lymphocyte population at certain stages of its development. The colonies differed markedly from one another in their curves of inhibition of rosette formation with DNP-erythrocytes by DNP-lysine, i.e., in the avidity of the B-cells of the given specificity. The colonies differed markedly also in the ratio between the volumes of the two clone groups studied (cells with anti-DNP and anti-BE Ig-receptors) with each other and with the total population of Ig-positive cells. These quantitative relationships were incompatible with the view that every B-cell contains every conceivable V-gene of the set, i.e., with the germ-line hypothesis of diversity of antibodies and receptors.

KEY WORDS: lymphocytopoiesis; clone formation; lymphocyte receptors; immunoglobulins.

Antibody synthesis in response to different antigens is known to begin at different times of individual development [12]. This is due to some extent to the asynchronous development of B and T cells [10, 14] and macrophages [2]. However, the possibility of asynchronous appearance of specialized B cells in individual development has also been demonstrated [7].

Clones of B cells in mice [11] appear on the 15th-16th day of embryogenesis. Before the 60th day of postnatal development the ratio between clones (based on the number of cells composing them), their ratio to the number of Ig-positive cells, and the distribution of cells of a given clone by avidity remain constant.

One paper has been published [14] in which clone formation was studied in a population consisting of progenies of a single stem cell taken from the liver of a 12-day mouse embryo. No specialized B cells were found in the primary splenic colony. They appeared in the spleen of the secondary recipient at different times for two different antigens (sheep's erythrocytes, polymerized flagellin).

The object of this investigation was to study lymphocytopoiesis and clone formation in the primary splenic hematopoietic colony.

EXPERIMENTAL METHOD

Mice (CBA) were irradiated in doses of 900-1000 R. A few hours later they were given an intravenous injection of $2.5\text{-}5.0 \cdot 10^5$ syngeneic bone marrow cells. The splenic hematopoietic colonies after 7-14 days, the recipients' spleens until the time of appearance of visible colonies, the intercolonial splenic tissue, the spleen of irradiated mice not receiving injections of bone marrow, and finally, a suspension of donors' bone marrow cells were investigated. In some experiments cells of the primary colony were injected into secondary recipients, and cells from secondary colonies into tertiary recipients. In other experiments, besides bone marrow, increasing doses of lymphocytes were injected into the recipients. Sheep, rat, and human erythro-

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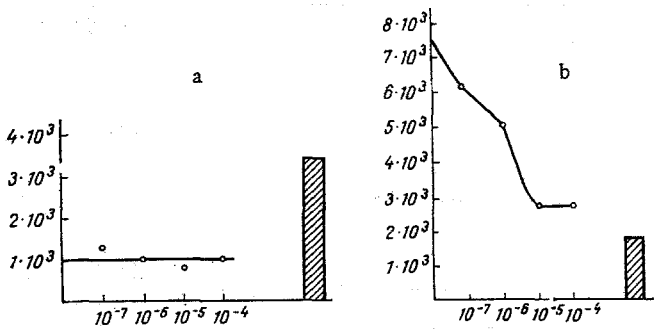

Fig. 1. Inhibition of DNP-RFC in splenic colony by increasing concentrations of DNP-ε-lysine: a) SE-RFC in colony are numerous(shaded column); DNP-RFC virtually absent; b) DNP-RFC with high avidity in splenic colony, number of DNP-RFC much greater than number of SE-RFC (shaded column). Ordinate, number of RFC; abscissa, concentration of DNP-lysine (in M).

cytes and DNP_{23}-ovalbumin, prepared with the use of 2,4-dinitrofluorobenzene [4], were used as antigens. The rosette formation test [9] was carried out with sheep's erythrocytes (SE) and with sheep's erythrocytes sensitized with DNP_{23}-ovalbumin (DNP-SE) [13]. Activity of rosette-forming cells (DNP-RFC) was investigated by using increasing concentrations (10^{-7}-10^{-4} M) of DNP-ε-lysine (Serva, West Germany).

The number of DNP-RFC was counted in not less than 20-80 · 10^4 cells in each sample. The error of the method was 8%. The Ig-positive cells were determined by the indirect luminescent antibody method with the necessary controls. For this purpose, and also to inhibit rosette formation, labeled and unlabeled rabbit antisera against mouse immunoglobulins and a fluorescent donkey serum against rabbit immunoglobulins (N. F. Gamaleya Institute of Epidemiology and Microbiology) were used. The sera were exhausted with liver powder and, in some experiments, with mouse thymocytes and erythrocytes and sheep erythrocytes.

EXPERIMENTAL RESULTS

From 300 to 500 SE-RFC and 400 to 600 DNP-RFC were counted per 10^6 cells in the spleen of CBA mice aged 2 months. The bone marrow contained 250 ± 58 cells forming rosettes both with SE and with DNP-SE.

Cells forming rosettes with sheep, rat, and human erythrocytes were found in the splenic colonies. For the quantitative analysis SE and DNP-SE were used.

The number of DNP-RFC in most colonies varied from 300 to 6000 and the number of SE-RFC from 400 to 5000 per 10^6 colony cells. The number of RFC with DNP-SE on the average for 40 colonies was 1900 ± 220 and the number of SE-RFC 1220 ±210 per 10^6 cells. The number of DNP-RFC in the same colonies inhibited by DNP-lysine (10^{-4} M) was 1070 ± 175. The mean number of DNP-RFC in the colonies evidently was a little greater than the number of SE-RFC (allowing for DNP-RFC with low avidity). The number of SE-RFC in 8 colonies was unusually high (5370 ± 415 per 10^6 cells). In five of them virtually no DNP-RFC could be detected (Fig. 1a).

Between the fourth and eighth days 150 ± 29 RFC per 10^6 cells were detected in the spleen of an irradiated mouse with SE and DNP-SE. No more than 100 RFC with SE andDNP-SE per 10^6 cells were found in the spleen of irradiated mice injected with $5 \cdot 10^5$ bone marrow cells between the third and sixth days. The corresponding number in the intercolonial tissue of the spleen between the eight and 14th days was 150 ± 30.

The number of DNP-RFC per 10^6 colony cells was reduced from 5500 to 2200 by DNP_{23}-ovalbumin (10^{-4} M); DNP-ε-lysine (10^{-4} M) reduced it to 2000, and rabbit antimouse antiglobulin serum (1 : 10) to 1100. The number of SE-RFC was reduced from 1100 to 300 by the action of antiglobulin serum. Ovalbumin (10^{-4} M) and normal rabbit serum did not reduce the number of DNP-RFC and SE-RFC.

The number of stem cells forming colonies was reduced tenfold in the tertiary recipients. They were injected with $1 \cdot 10^7$ cells from the secondary recipients. In ten colonies from tertiary recipients the mean number of DNP-RFC was 4900 per 10^6 colony cells. Three groups of primary colonies were tested at the same time. The mean number of DNP-RFC was 3725 in the first group (11 colonies), 3100 in the second group (five colonies), and 7400 in the third group (five colonies) per 10^6 colony cells (without inhibition by DNP-lysine).

A sharp increase in the number of colony cells transplanted into the tertiary recipients thus did not cause any evident increase in the number of DNP-RFC in the colonies formed in the tertiary recipients.

After injection of $1 \cdot 10^6$, $2 \cdot 10^6$, or $10 \cdot 10^6$ lymph node cells in addition to $5 \cdot 10^6$ bone marrow cells into the recipients the number of DNP-RFC and of SE-RFC in the 8-day colonies was not increased.

Starting from the 6th day DNP-RFC, SE-RFC, and Ig-positive cells were found regularly in the small colonies. Minimal numbers of Ig-positive cells were found (0.06 ± 0.065%) in the spleen of the irradiated mouse. On the 8th day their number in the colonies varied from 0 to 2.45% (0.91 ± 0.28) and on the 12th day from 3.1 to 45.9% (23.12 ± 8.51). The number of Ig-positive cells found in the bone marrow suspension was 1.17 ± 0.27%, in the thymus 0.61 ± 0.29%, and in the intact spleen 38.6 ± 3.4%. In 40 colonies the distribution of DNP-RFC by avidity was studied. Nearly all colonies differed significantly from each other in their DNP-RFC inhibition curves (Fig. 1). No difference was found between the 8th-9th day colonies and the 12th-13th day colonies. The spectrum of avidity of DNP-RFC was wide in 7 colonies and very wide in 2 colonies, i.e., they contained cells of high avidity. The ratio between the number of DNP-RFC and SE-RFC and the number of Ig-positive cells was studied in 7 colonies. No regular relationship could be found between them. For instance, 200 SE-RFC and 700 DNP-RFC (per 10^6 cells) were found in one colony containing 2.3% Ig-positive cells (1.6% SE-RFC and 5.5% DNP-RFC among the Ig-positive cells). In another colony the ratio was different: 500 SE-RFC, 1200 DNP-RFC, and 1.1% of Ig-positive cells (1% of SE-RFC and 2.4% of DNP-RFC among all Ig-positive cells).

At the end of the 1960s an unambiguous answer was obtained by the chromosomal marker method to the question of whether any other cells transplanted into the recipient with the suspension of donor's bone marrow proliferate in a splenic hematopoietic colony developing from a single stem cell [5]. It was shown by the same method [14] that the lymphopoietic colony arises from the donor's stem cell and contains virtually no admixture of recipient's lymphocytes.

The results of the present experiments in which 20 times that number of colony cells were transplanted into tertiary recipients and of experiments involving "subculture" of increasing doses of lymphocytes from lymph nodes, together with the results of investigations cited above, suggest that the hematopoietic splenic colony does not contain any significant admixture of donor's and recipient's B cells.

However, an unambiguous answer to this question requires further intensive investigations, including by the methods of chromosomal or antigenic markers.

The principle of constancy of the ratio between the volumes of groups of clones in individual development [11] reflects the random character of clone formation. It must be postulated from the general propositions of the clonal selection theory that, beginning from a certain starting point, the hypothetical inducer of lymphocytopoiesis expresses all V genes contained in the given population of differentiating B cells with equal probability [8].

Since, as a rule, only one V gene can be expressed in one B cell, the regular quantitative ratios between groups of clones described in "averaged" populations of adequate size [11] must reflect the corresponding quantitative ratios between the V genes in these populations of B lymphocytes.

Positive selection of B cells at particular stages of their development is evidently unimportant [10, 11]. The question of vegetative selection [3] remains open.

Our initial assumption (see above) was that in the hematopoietic colonies studied there are progenies of a single stem cell, free from any significant contamination with the donor's or recipient's lymphocytes. No regular quantitative relationships were found between the groups of clones and Ig-positive cells in the colonies. The colonies varied sharply in avidity of DNP-RFC, in the ratio between the numbers of DNP-RFC and SE-RFC and, finally, in the ratio between the numbers of SE-RFC and DNP-RFC and the number of Ig-positive cells. The significant differences in avidity between the B cells reflect differences in the affinity of their receptors [1, 6] and, evidently, differences in the structure of the coding V genes. The fractions of DNP-RFC and SE-RFC were approximately not more than 1% of the number of Ig-positive cells in the colony. Assuming the equally probable expression of V genes during lymphocytopoiesis and that in each cell there is every conceivable V gene of the set (the germ-line theory), the likelihood of appearance of colonies with highly avid DNP-RFC, like the probability of appearance of colonies in which, for example, DNP-RFC are virtually absent or present in very small numbers (7 of 40 colonies), is extremely small, for the clone volumes (based on the number of cells) ought not to deviate from the mean by more than 3σ with a probability of 0.997:

$$\sigma = \sqrt{Npq},$$

where σ is the standard deviation of the number of cells in the clone; N the number of Ig-positive cells in the colony; $p = n_{mean}/N$ is the probability of expression of the V gene of the particular specificity; n_{mean} is the mean number of cells of that specificity among all colonies tested; $q = 1 - p$.

The following basic assumption was made above: At a certain stage of ontogeny the quantitative ratios between clones of B lymphocytes reflect quantitative ratios between the corresponding V genes in the given population. If this assumption is accepted, the sharply varying quantitative ratios discovered in these experiments between groups of clones and Ig-positive cells in the individual colonies are incompatible with the view that every B cell contains every conceivable V gene of the set, i.e., they are incompatible with the germ-line theory of antibody diversity. The model used is extremely suitable for further detailed quantitative analysis of this problem.

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ABILITY OF LYMPHOCYTES STIMULATED BY PHYTOHEMAGGLUTININ in vitro TO PARTICIPATE IN THE GRAFT VERSUS HOST REACTION

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Lymph node cells from CBA mice stimulated for 2 h by phytohemagglutinin were more able, whereas cells cultivated for 44 h with phytohemagglutinin were less able, than intact lymph node cells to participate in the graft versus host reaction when injected into sublethally irradiated (CBA × C57BL/6)F_1 hybrids. Syngeneic lymphocytes and killed allogeneic lymphocytes cultivated in the same way, like phytohemagglutinin itself, had no such action.

KEY WORDS: colony-forming units; phytohemagglutinin; lymphocyte; allogeneic transplant.

The ability of lymphocytes of the peripheral blood and lymphoid organs to proliferate in vitro under the influence of antigen or mitogen has been widely used in experimental and clinical research [7, 8, 10]. The dynamics of many biochemical and morphological processes in stimulated lymphocytes has been studied in

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