# LOCAL HEMOLYSIS IN AGAR AS A TEST OF INACTIVATION OF NONSYNGENETIC* STEM CELLS

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After incubation of spleen cells of C57BL and CBA mice in vitro these cells loss their ability to form zones of hemolysis in agar, i.e., proliferation of cells sensitive to the antigen (sheep's erythrocytes) is suppressed. Irradiation of one of the components of the cell suspension (CBA cells) abolishes this phenomenon.

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A previous investigation [1] showed that interaction between cell suspensions of lymphoid tissues of two different genotypes for 1 h at 37° leads to considerable or complete inactivation of colony-forming activity of the stem cells as revealed by the method of Till and MacCulloch [2]. The inactivation index in mixtures of spleen cells of C57BL and CBA mice ($2 \cdot 10^6$ of each) was 69.9-85.4%, compared with 100% in mixtures of spleen cells of C57BL mice and lymph gland cells of CBA mice.

The present investigation was carried out to determine whether this phenomenon can be analyzed not only by the method of exogenous intraplenic colonies suggested by Till and MacCulloch, but also by counting the number of antibody-forming cells by the method of formation of zones of hemolysis in an agar plate [3].

## EXPERIMENTAL METHOD

The scheme of the experiments, using the technique described by Jerne and Nordin [3], is illustrated in Fig. 1. In principle, the scheme is a repetition of that published previously, using exogenous colonies

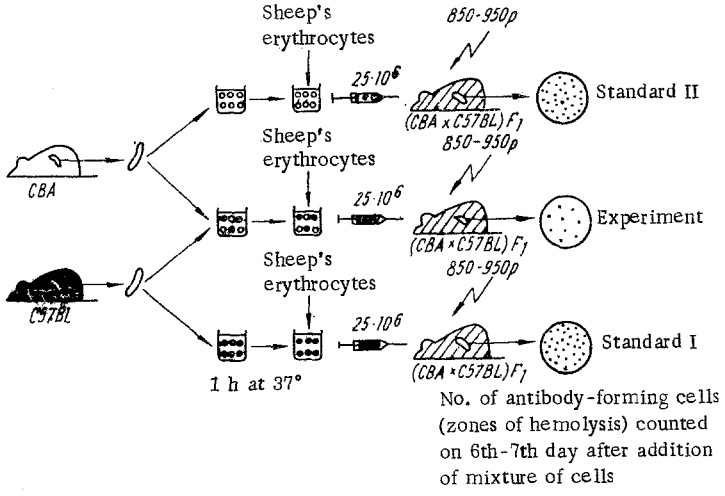


Fig. 1. Scheme of application of Jerne's method for studying the phenomenon of inactivation of nonsyngenetic stem cells.

*In accordance with the new immunologic terminology, genetically identical organisms, tissues, or cells are described as syngenetic, while the opposite term "nonsyngenetic" implies absence of their genetic identity.

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for analysis of the phenomenon [1]. The difference is that before transplantation of cells into lethally irradiated $F_1$ hybrids, sheep's erythrocytes were added to the cell suspensions. This antigen stimulated cells sensitive to it, and their progeny can be detected as antibody-producing cells.

The donors, intact C57BL and CBA mice, were sacrificed. A suspension of cells in medium No. 199 was prepared from the spleen of the animals of each line by chopping it with scissors and passing it through a syringe. The cells were washed twice in medium No. 199, after which the total number of nucleated cells per ml of suspension was counted. By staining with trypan blue the number of living cells in the suspension was determined. Their number was adjusted to $50 \cdot 10^6$/ml. The spleen cells of the C57BL (standard I) and CBA mice (standard II), and a mixture of equal numbers of spleen cells of mice of both lines (experiment) were then incubated for 1 h at 37°. At the end of incubation, washed sheep's erythrocytes were added to the suspensions in numbers of $65 \cdot 10^6$–$70 \cdot 10^6$ erythrocytes per recipient. The resulting suspensions were injected in a dose of $25 \cdot 10^6$ nucleated cells of each genotype into the lateral tail vein of $F_1$ recipient hybrids (C57BL × CBA) lethally irradiated with $Co^{60}$ $\gamma$ rays in a dose of 850–950 R. The spleen cells were irradiated in vitro with the same apparatus in a dose of 10,000 R. The recipients were sacrificed 6–7 days later and the number of hemolysin-producing cells in their spleen was counted. A suspension of cells was made from the spleen of each recipient separately and the number of nucleated cells per ml of suspension was counted. A predetermined number of spleen cells was mixed with 1.4% liquid (at 40–45°) Difco agar together with sheep's erythrocytes and poured into Petri dishes, two dishes for each recipient. After the agar had solidified, the dishes were incubated for 1.5 h at 37°, after which complement (dried guinea pig serum from the I. I. Mechnikov Institute of Vaccines and Sera) was added to the agar plates in a dilution of 1 : 5. After further incubation for 1 h at 37° the number of zones of hemolysis in the agar was counted visually. The titers of agglutinins and hemolysins against sheep's erythrocytes were determined in the serum of all the recipients.

In some experiments the previously described method of counting the number of colony-forming cells of donor origin settling in the recipient's spleen as described by Till and MacCullouch [2] was also used. In this case, each recipient, irradiated in the same dose, received an injection of $2 \cdot 10^6$ intact spleen cells of C57BL mice (standard I), or $2 \cdot 10^6$ spleen cells of CBA mice (standard II), or $4 \cdot 10^6$ cells from C57BL and CBA mice in a ratio of 1 : 1 (experiment). In this case no sheep's erythrocytes were added. On the 8th–9th day the recipients were sacrificed, their spleens were fixed in Bouin's fluid, and the number of colonies in the spleen was then counted.

In all the experiments the inactivation index (II) was calculated by the formula:

$$\text{II} = 100 - \frac{X \cdot 100}{Y},$$

where X is the actual number of zones of hemolysis calculated per $10^6$ spleen cells tested or the actual number of colonies in the spleen, and Y represents the expected number of zones of hemolysis or colonies obtained by simple summation of the results of standard I and standard II groups (Fig. 1).

## EXPERIMENTAL RESULTS

The experimental results are given in Table 1. Injection of $25 \cdot 10^6$ spleen cells of C57BL mice mixed with sheep's erythrocytes led after 6 days to the accumulation of 7.6, and after 7 days 29.42 antibody-forming cells in their spleen (calculated per million spleen cells). The same number of spleen cells from CBA mice gave 5.05 and 61.69 (group No. 2 of experiments B and A) antibody-forming cells respectively. A mixture of cell suspensions from C57BL and CBA mice under the same conditions led to the accumulation of only 1.1 (6th day) and 11.3 (7th day) antibody-forming cells (group No. 3 of experiments B and A). The inactivation index, calculated at these different times, was 74.9 and 82.6%. A similar inactivation index (75.9%) also was recorded when inactivation of the stem cells was investigated by the method of Till and MacCulloch (experiment C).

Experiments B' and C' showed that the phenomenon of inactivation of stem cells is not manifested if one of the components of the mixture is irradiated. Irradiation of spleen cells of CBA mice in a dose of 10,000 R led to practically complete inactivation of their proliferating ability (group No. 2 in experiments B' and C'). Interaction of these cells with intact spleen cells of C57BL mice had no effect on the number of stem cells or of the number of them sensitive to antigen. A mixture of intact C57BL cells with irradiated

TABLE 1. Experimental Detection of the Phenomenon of Inactivation of Nonsyngenetic Stem Cells (M±m)

| Experiment | Group No. | Cells used | No. of recipients | No. of cells injected per recipient × $10^6$ | Mean No. of antibody-forming cells in recipient's spleen (per million spleen cells) | Mean No. of colonies in spleen by Till and MacCulloch's method | Expected No. of cells (1/2 of sum of standard I + standard II) | Inactivation index (II) |
|---|---|---|---|---|---|---|---|---|
| A | 1 | C57BL (Standard I) | 12 | 25 | 29,43±5,98 | | | |
| | 2 | CBA (Standard II) | 6 | 25 | 61,59±13,2 | | | |
| | 3 | C57BL+CBA (Experiment) | 12 | 12,5+12,5 | 11,3±1,9 | | 45,51±2,88 | 74,9% |
| B | 1 | C57BL (Standard I) | 5 | 25 | 7,60±2,67 | | | |
| | 2 | CBA (Standard II) | 5 | 25 | 5,05±0,45 | | | |
| | 3 | C57BL+CBA (Experiment) | 5 | 12,5+12,5 | 1,1±0,38 | | 6,33±0,71 | 82,6% |
| B′ | 1 | C57BL (Standard I) | 5 | 25 | 7,60±2,67 | | | |
| | 2 | CBA, Irradiated 10 000 *p* (Standard II) | 8 | 25 | 0,13±0,12 | | | |
| | 3 | C57BL+CBA (Experiment) | 12 | 12,5+12,5 | 3,6±1,2 | | 3,86±0,62 | no inactivation |
| C | 1 | C57BL (Standard I) | 25 | 2 | | 17,56±0,92 | | |
| | 2 | CBA (Standard II) | 22 | 2 | | 4,5 ±0,45 | | |
| | 3 | C57BL+CBA (Experiment) | 25 | 2+2 | | 5,3±0,41 | 22,06±0,88 | 75,9% |
| C′ | 1 | C57BL (Standard I) | 15 | 2 | | 15,7±0,82 | | |
| | 2 | CBA, Irradiated 10 000 *p* (Standard II) | 14 | 2 | | 0,14±0,08 | | |
| | 3 | C57BL+CBA (Experiment) | 9 | 2+2 | | 15,5±1,91 | 15,8±1,05 | no inactivation |
| | 4 | 0 | 11 | 0 | | 0,2 | | |

Note. In experiment A recipients were irradiated in a dose of 590 R; results read on 7th day; in experiments B and B′ recipients were irradiated in a dose of 850 R and results were read on the 6th day; in experiments C and C′ recipients were irradiated in a dose of 850 R and results were read on the 8th day.

CBA cells caused the accumulation of the same number of antibody-forming cells or colonies in the spleen as that produced by the unirradiated component only, i.e., by C57BL cells (group No. 3 in experiments B′ and C′). In this case inactivation was absent.

The phenomenon of inactivation of nonsyngenetic stem cells, as described above, taking place during combined incubation of intact lymphoid cells of mice of two inbred lines is therefore observed not only by counting the number of intraplenic colonies. It can also be analyzed by Jerne and Nordin's method of local hemolysis in agar. In this case, however, it is possible that different stem cells are concerned.

The manifestation of this phenomenon evidently requires interaction of viable cells, because irradiation (10,000 R) of one component of the cell mixture suppresses the phenomenon.

It may be considered that the phenomenon of inactivation of nonsyngenetic stem cells may play an important role in maintenance of genetic homeostasis of the macroorganism (suppression of mutant forms of somatic cells). In this connection the results of these experiments suggest that this mechanism of identification of mutations is damaged in the irradiated organism.

## LITERATURE CITED

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