DETERMINATION OF ANTIBODIES AND NONSPECIFIC IMMUNOGLOBULINS FORMED IN A SINGLE CELL

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Immunization of animals is known not only to lead to antibody formation, but also to sharply increase the synthesis of nonspecific immunoglobulin (NIG) [4]. It was postulated a few years ago that both these proteins may be synthesized in the same cells [3, 10]. The discovery of many cells containing both antibodies and NIG simultaneously was subsequently reported [2, 5]. However, results obtained by the immunofluorescence and immunoenzymic methods used in these investigations cannot be unambiguously interpreted.

The object of this investigation was to test this hypothesis by the use of a fundamentally new approach.

EXPERIMENTAL METHOD

The suggested method is based on identification of products secreted by cells of the immune spleen. The cells are applied to millipore filters [9], placed on a layer of native sheep's red blood cells (SRBC) embedded in agarose, or on a layer containing SRBC sensitized by antibodies against immunoglobulins produced by the cells. The localization of antibody-forming cells (AFC) and immunoglobulin-forming cells (IFC) could be revealed by direct or indirect [6] and reverse [7] plaque techniques. If immobilized antigen, specifically binding antibodies synthesized by the cells was introduced between the filter containing the cells and sensitized SRBC, all zones of hemolysis (plaques) discovered under the filter must belong only to NIG producers (NIFC). By transferring the filters with the cells from the layer with native SRBC to a layer with sensitized SRBC, and comparing the location of the plaques on these layers, it was thus possible to determine whether a cell synthesized only antibodies or both antibodies and NIG.

Female BALB/c mice weighing 14-16 g, immunized twice with SRBC at an interval of 2-4 weeks (0.15 ml of a 20% suspension, intraperitoneally, each time) were used. The spleen was removed on the 4th day after reimmunization and a cell suspension was prepared from it.

Cells producing antibodies against SRBC and NIG were detected by a modified method [9]. The cells were washed three times with Eagle's medium (Institute of Poliomyelitis and Viral Encephalitis, Academy of Medical Sciences of the USSR), cooled to 0°C, and applied to No. 5 membrane ultrafilters with a pore diameter of 0.4 μ (Experimental Ultrafilters Factory, Academy of Medical Sciences of the USSR). To apply the cells to the filter, it was placed in an apparatus usually used for preparation of radioactive targets, on two or three layers of filter paper. Cold Eagle's medium containing 10% embryonic calf serum (ECS) from Gibco, Bio-Cult, Scotland, in a volume of 10 ml, was poured into the top part of the apparatus, and the required number of spleen cells, washed with Eagle's medium, in a volume of 0.1 ml was added. The cells were deposited on the filter by a weak flow of fluid for 1.5-2 min. The filter with cells deposited on it was placed in a petri dish (60 mm) on a layer of SRBC embedded in agarose.

The agarose (from Bio-Rad, USA) was made up in Eagle's medium (Industrial Biological Laboratories, USA), containing 10% ECS. To obtain 0.75% agarose, a 1.5% solution of agarose

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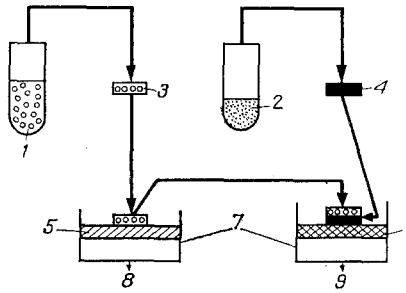

Fig. 1. Scheme of experiments to detect secretion of antibodies and NIG by the same cells. 1) Suspension of spleen cells; 2) suspension of SRBC; 3) cells on millipore filter; 4) SRBC on millipore filter; 5) native SRBC; 6) sensitized SRBC; 7) 0.75% agarose; 8) detection of AFC; 9) detection of NIFC.

in water and double-strength Eagle's medium (2.12 g of dried concentrate to 100 ml water), pH 7.2-7.4 (adjusted with the aid of 10% soda solution), were prepared. To the required volume of double-strength Eagle's medium (V) ECS was added to a final concentration of 10%; the mixture was heated to 45°C in an ultrathermostat and mixed with volume V of melted 1.5% agarose.

The 0.75% agarose thus prepared was poured into petri dishes (2 ml per dish) and into test tubes heated to 45°C (0.5-0.75 ml per tube). The tubes were left in the ultrathermostat at 45°C, whereas the dishes, after solidification of the agarose underlayer, were incubated at 37°C.

To detect cells synthesizing 7S-antibodies (Jerne's indirect method), native SRBC were used; to detect cells producing NIG, SRBC sensitized with rabbit antibodies against mouse IgG were used.

Antibodies against mouse IgG were isolated from the serum of immunized rabbits with the aid of a specific immunosorbent containing mouse IgG conjugated with aminocellulose [1].

IgG were isolated from the serum of BALB/c mice with a transplanted MOPC-21 plasmacytoma by the method in [8].*

SRBC, native or sensitized with antibodies, were washed three times with 0.9% NaCl solution, a 20% suspension was prepared, and this was transferred in a volume of 0.03 ml to test tubes containing agarose, heated to 45°C. The well-mixed SRBC suspension in agarose was quickly poured onto a dish with an underlayer and the dishes were allowed to solidify at room temperature (for 5 min).

To find cells producing antibodies and NIG simultaneously, the filter with cells applied to it was placed initially on a layer of native SRBC embedded in agarose, and incubated in a humid atmosphere of air with 10% CO_2 at 37°C for 30-60 min. At the end of incubation the oriented filter with the cells was transferred to a layer of sensitized SRBC, embedded in agarose, and incubated as described above for a further 2-3 h. To eliminate antibodies against SRBC produced by the cells, a millipore filter on which was applied a suspension of native SRBC (0.03 ml of a 10-20% suspension per filter) was introduced between the filter with the cells and the layer of sensitized SRBC. The scheme of the experiment is illustrated in Fig. 1.

At the end of incubation the filters were removed and the dishes with native and sensitized SRBC were treated with 2 ml of rabbit antiserum against mouse IgG (in a dilution of 1:100) or with a solution of anti-IgG-antibodies (30 μg/ml), and incubated for 1 h at 37°C. The antibodies were then poured off, the surface of the agarose was sprinkled with 0.9% NaCl, 2 ml of a solution of complement was poured into each dish (guinea pig serum in a dilution of 1:10), and the dishes were incubated for a further 1 h at 37°C, when the results were read. The determinations were carried out in two or three parallel tests.

EXPERIMENTAL RESULTS

Incubation of the cells on millipore filters under the conditions described above revealed approximately the same numbers of AFC and IFC as incubation of the cells directly in a layer of native or sensitized SRBC. For instance, the number of 7S AFC during the secondary immune response, determined by Jerne's method, averaged 2100 ± 900 per 10^6 spleen cells, whereas the number of AFC revealed by applying the cells to millipore filters was 2200 ±

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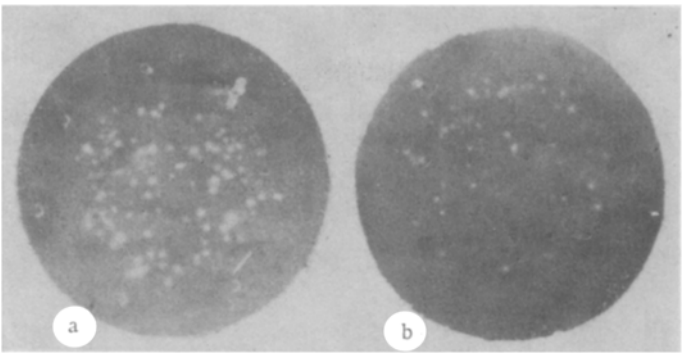

Fig. 2. Zones of hemolysis (plaques) revealed beneath filters with spleen cells of mouse immunized with SRBC. a) During incubation of filters with cells on layer of agarose containing native SRBC (detection of 7S-AFC); b) on incubation of filters with cells on layer of agarose containing sensitized SRBC (detection of IFC).

700 per 10^6 cells (experiments on 15 and 10 mice, respectively). The corresponding figures for IFC were 4600 ± 600 and 3800 ± 800 per 10^6 cells (experiments on 10 and six mice, respectively). The number of AFC was thus about 50% of the total number of spleen cells producing immunoglobulins. The number of plaques detectable beneath the filters was proportional to the doses of cells applied to the filter. The plaques were bright and clear, although they varied considerably in size (Fig. 2).

After transfer of filters with cells from one dish to the other the number of plaques detected fell considerably in most experiments. This decrease was observed both during successive transfers from native to native SRBC and during transfers from native to sensitized SRBC.

Since both cells synthesizing antibodies and cells synthesizing NIG were present in the method used to determine the number of IFC, in order to differentiate one from the other, an interlayer of SRBC applied to millipore filters and playing the role of adsorbent, taking up antibodies against SRBC, was placed between the cells and the layer of sensitized SRBC.

The "working capacity" of this red blood cell sorbent was tested in a special experiment. Filters with different doses of SRBC and of donkey RBC, which are known not to cross-react with SRBC at the antibody level, were prepared. The dose for SRBC varied from 0.03 ml of a 2.5% suspension to 0.03 ml of a 20% suspension, and the dose for donkey RBC varied from 0.03 ml of the 5% suspension to 0.03 ml of a 20% suspension. The sorbent prepared from donkey RBC, even in their highest concentration, was found to cause virtually no decrease in the number of plaques detectable beneath the filters (11-12 compared with 13-14 in control samples incubated without the "interlayers"). Meanwhile, even the minimal dose of SRBC used as the sorbent led to a sharp decrease in the number of AFC detected (0-2 plaques compared with 13-14). The use of a layer of a 10-20% suspension of SRBC thus virtually guaranteed the complete uptake of antibodies produced by single cells. It was then possible to move on to experiments to compare the location of zones of lysis on the dishes with native and sensitized SRBC.

Experiments with transfer of filters with cells from native to sensitized SRBC, with the use of filters to which were applied a 20% suspension of SRBC as interlayers, showed (Fig. 3a, b) that after application of 50-80 × 10^3 immune spleen cells on a layer of native SRBC to the filter, 80-100 plaques were counted. When these filters were transferred to sensitized SRBC, plaques also were revealed on them, but their total number was sharply reduced, their outlines were more indistinct, and they were much less bright than after transfer of filters without "interlayers." The overwhelming majority of plaques detected on sensitized SRBC were located in different places from the plaques on native SRBC. Meanwhile, after these transfers it was usually possible to find between one and three plaques whose positions on the native and sensitized SRBC coincided (indicated by arrows in Fig. 3).

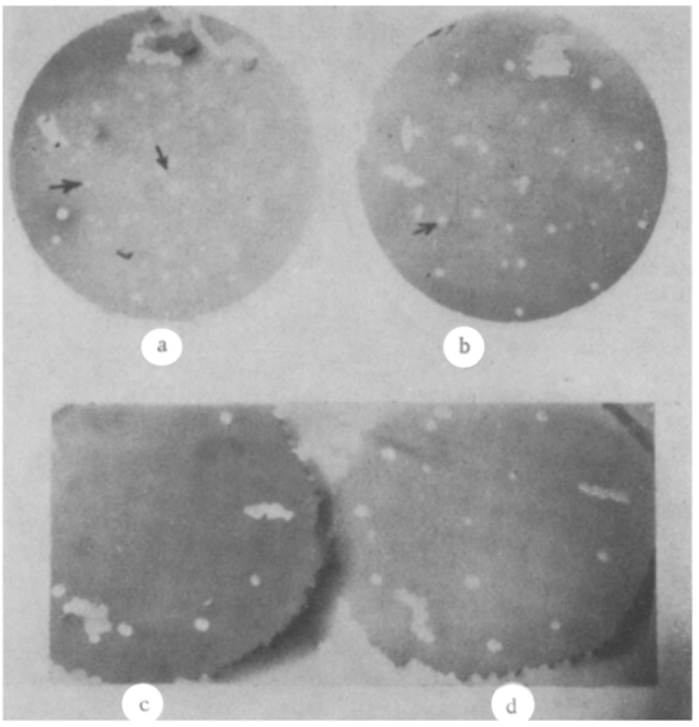

Fig. 3. Zones of hemolysis detected beneath filters in experiments with transfer of filters from native SRBC (a, c) to sensitized SRBC (b, d), with the use of an "interlayer" composed of SRBC applied to millipore filters (detection of AFC and NIFC). Number of cells applied to filters 50×10^3 (a, b) and 3×10^3 (c, d), respectively.

It will be evident that when 50×10^3 cells are applied to a filter 2 cm in diameter, their density is so great that, even if the localization of the plaques on dishes with native and sensitized SRBC was the same, it could not be asserted that they were definitely formed by the same cell. Accordingly, the next step was to carry out experiments in which far fewer cells were applied to the filters. The results of one such experiment, in which 3×10^3 immune spleen cells were applied to each filter are given in Fig. 3c, d. Under these conditions, five to seven plaques could be counted on dishes with native SRBC and three to four plaques on dishes with sensitized SRBC; the localization of the two did not coincide.

It can be concluded from these results that during the secondary immune response at least the majority of AFC does not synthesize nonspecific immunoglobulins. The discovery of between one and three coincident zones of hemolysis on layers of native and sensitized SRBC, following application of large numbers of cells to the filters, may perhaps be due to the difficulty of accurately differentiating the positions of the plaques under these conditions or to the presence of cells lying close together, of which one produces antibodies and the other NIG. Meanwhile, the presence of such "double producers" cannot be ruled out in general on the basis of these experiments. All that is clear is that their number is extremely small and that it is impossible to explain the increase in NIG synthesis observed during immunization by their formation in the same cells as those which produce antibodies.

It must be pointed out that the suggested method can be used to detect the simultaneous formation and secretion, by single cells, of any compound against which antibodies can be obtained. The method is relatively simple, highly sensitive, and specific. The results are reproducible.

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IMMUNOLOGIC TOLERANCE TO SURFACE POLYSACCHARIDES OF MENINGOCOCCI OF SEROGROUPS A AND C

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Induction of a state of specific immunologic areactivity to bacterial antigens of polysaccharide nature is a difficult experimental task. For instance, in order to induce immunologic tolerance to the polysaccharide Vi-antigen of *Salmonella typhi* the method used is to treat the animals with antilymphocytic sera [4] or cyclophosphamide [7] in conjunction with injection of relatively high doses of antigen — 200 μg or more. To induce tolerance to levan [9] or the polysaccharide of type III pneumococci [8], doses 250-1000 times larger than the optimal immunizing dose are required.

It was shown previously during a study of the dependence of the immune response of mice on the dose of meningococcal polysaccharides, that the response of animals receiving from 50 to 1000 μg of these antigens was considerably lower than that of mice receiving antigens in doses of 0.005 to 0.5 μg [5].

The object of this investigation was to study whether inhibition of the immune response observed is in fact immunologic tolerance and, if so, to study some particular features of this phenomenon.

EXPERIMENTAL METHOD

Experiments were carried out on CBA, AKR, and (CBA × C57BL/6)F_1 mice and on noninbred albino mice of both sexes weighing 18-20 g. Surface polysaccharides of meningococci of serogroup A (PA), which is a polymer of N-acetyl-O-acetyl-mannosamine phosphate, and polysaccharide from meningococci of serogroup C (PC), which is a polymer of N-acetyl-O-acetyl-neuraminic acid, were used as antigens. Both antigens, which were obtained from the Moscow Research Institute of Epidemiology and Microbiology, Ministry of Health of the RSFSR, by a modified method of Gotschlich et al. [3], had molecular weights of about 100,000.

To induce an immune response, antigens were injected intravenously into the mice and, on the 4th day, the number of antibody-forming cells (AFC) was counted in the spleens by a modification of the standard passive local hemolysis-in-gel technique [6]. The main features of the method were described previously [5]. At the same time as AFC were detected, the antibody titers in the animals' sera were determined by the passive hemagglutination test (PHT).

In some experiments, specificity of tolerance was verified by the use of the polysaccharide Vi-antigen of *S. typhi*. To detect AFC against Vi-antigen, the passive local hemol-

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