Autoradiographic Plaques for the Detection of Antibody Formation to Soluble Proteins by Single Cells

Abstract. *Single cells that synthesize antibody to soluble proteins can be detected and counted by an autoradiographic procedure. The method is based on the specific binding of I^{131}-labeled antigen to the antibody which has been produced by and has diffused around cells in an agarose medium and which has been precipitated by an antibody to gamma globulin. The antigen-binding zones appear as dark spots on high-speed x-ray film exposed to the dried agarose preparations.*

Since Jerne and Nordin (*1*) and Ingraham and Bussard (*2*) described localized hemolysis in gels, many attempts have been made to extend the use of this technique to antigens other than red cells (*3*). These methods depend on the lysis of red cells or bacteria mediated through the complement system and therefore record a phenomenon secondary to the interaction between antigen and antibody. They are restricted also to the use of a small number of particulate antigens and to the enumeration of cells synthesizing 19*S* antibody. Baker *et al.* (*4*) tried to overcome some of the disadvantages of these techniques by using bentonite particles covered with antigen. Cells that synthesize antibody specifically bind the particles and can be enumerated. Our method permits the detection of antibody-synthesizing cells based on the primary interaction between antibody and I^{131}-labeled antigen, and it provides a quantitative measure of cells forming specific immunoglobulins of all types. The principle of the method is similar to that on which radioimmunoelectrophoresis (*5*) is based: antibody, when precipitated in agar by an antibody to γ-globulin, binds specific antigen labeled with radioactive iodine. The complex formed becomes visible by exposure to a photographic or x-ray film.

Portions (1 mg) of bovine serum albumin (BSA) were labeled with 5 mc of carrier-free I^{131} (*6*). To eliminate free iodide and to obtain a relatively pure antigen, the labeled protein was dialyzed at 4°C overnight against 0.1*M* phosphate buffer, *p*H 7.0, and then passed through a Sephadex G-25 (fine) column (40 by 2 cm). A narrow fraction of the first and major peak obtained from the column was used; its specific gravity varied from 1 to 2 μc per microgram of BSA, and the antigen as used was adjusted to 1 μg per milliliter.

Antibody to rat γ-globulin was prepared by immunization of rabbits. The rat γ-globulin was obtained by preparative electrophoresis on Pevikon (*7*). The antiserum detected immunoglobulin G (γG), M (γM), and an unidentified β-globulin by immunoelectrophoresis of whole rat serum. A γ-globulin fraction was separated from the antiserum by precipitation with ammonium sulfate at 50 percent saturation and brought to a concentration of 20 mg per milliliter of protein.

Male Lewis rats were immunized by a single injection of BSA (2 mg/ml) in complete Freund's adjuvant into the four footpads and two subcutaneous sites. Some animals received booster injections of 1 mg of BSA at later intervals; the injections were made in four subcutaneous sites. Immune lymphoid cells were obtained from the lymph nodes draining the injected footpads and in some cases from thoracic duct lymph. The cells were washed twice in Earle's balanced salt solution by alternate centrifugation and suspension. They were finally dispersed in Eagle's minimal essential medium without bicarbonate, counted, and brought to the desired cell concentration.

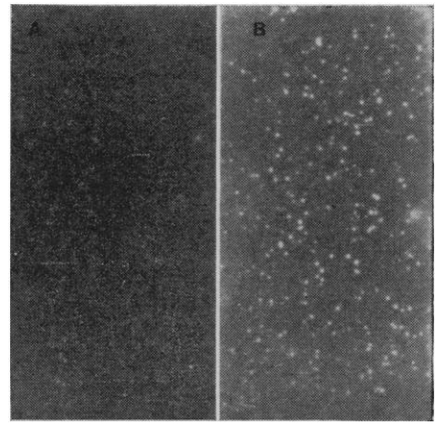

Fig. 1. Autoradiograph on x-ray film of two slides on which were plated lymph node cells from a normal rat (A), and from a rat immunized 12 days before with 2.0 mg of bovine serum albumin (BSA) in complete Freund's adjuvant (B). On each slide, 1×10^6 cells were plated. Autoradiographic plaques appear white in the print but are gray to black on the x-ray film.

Agarose (*8*) was made up as a 0.5 percent solution in Eagle's medium without bicarbonate and distributed in 0.5-ml portions, while it was hot, among small test tubes kept at 44°C.

Regular microscope slides with frosted ends were coated with 0.1 percent agarose in distilled water. To 0.5 ml of agarose in tubes, 0.1 ml of cells was added (2 to 10 $\times$ 10^6 cells/ml). The mixture was quickly shaken to achieve a homogeneous suspension and poured on the nonfrosted part of the microscope slide. The above volumes produced an even layer of agarose that hardened quickly at room temperature. The slides were incubated for 5 hours at 37°C in a humidifying chamber. They were then placed, agarose face-down, on special Lucite racks which allowed contact between the agarose layer and a fluid phase containing rabbit antibody to rat γ-globulin, 100 μg per milliliter (*9*). The slides were covered with antiserum, kept at 4°C for 24 hours, and subsequently washed for 24 hours with four changes of ice-cold phosphate-buffered saline (PBS), *p*H 7.0 (PBS 7). At the end of this period, the slides were placed in other Lucite racks, flooded with I^{131}-labeled BSA for 24 hours at 4°C, and finally washed for 72 hours with three or four changes daily of cold PBS 7. The preparations were covered with bibulous paper and dried overnight at 37°C. At this point, KK industrial x-ray film (*10*) was firmly pressed against the agarose surface in the dark and exposed for 24 hours. The film was developed for 5 minutes in DK-50 developer (*10*), fixed, and washed as usual.

Presumably, the γ-globulin which was synthesized by the cells in agarose during the 5-hour incubation period diffused around the cell and was precipitated in an excess of rabbit antibody to rat γ-globulin. The precipitate was invisible grossly but could be seen after labeled antigen became fixed to the precipitated rat γ-globulin and produced a cluster of grains in the photographic emulsion. The sites of cells that synthesized antibody appeared grossly on the x-ray film as uniformly round spots (autoradiographic plaques) which ranged from 0.5 to 1.0 mm in diameter and varied from dark gray to tan, depending on the number of grains present (Fig. 1). When the number of cells which synthesized antibody was high, there was occasional overlap between adjacent plaques, and accurate counting was difficult. To avoid this,

Table 1. Proportionality between the number of autoradiographic plaques on x-ray film and the number of immunized cells plated. Thoracic duct lymphocytes of rats were harvested 20 days after an immunizing dose of 2 mg of bovine serum albumin (BSA) and 13 days after a second dose of 1 mg of BSA. Draining lymph node cells were harvested 12 days after immunization with 2 mg of BSA. Eight slides were prepared for each cell concentration.

Cells plated per slide (No.)	Plaques per slide (mean value)	Plaques per 10^6 cells (calculated)
Thoracic duct cells		
0.02×10^6	28	1400
0.2×10^6	231	1155
1.0×10^6	931	931
Draining lymph cells		
0.2×10^6	168	840
1.0×10^6	910	910

cells were plated at two or three different cell concentrations.

Autoradiographic plaques on the x-ray film were larger than a single cell and covered an area which contained several nucleated elements. To ascertain whether a plaque corresponded to a single cell, the slides were scored on their undersurfaces at the sites of the plaques in the x-ray film. The x-ray film was removed; the slides were dipped in NTB-2 track emulsion (*10*), exposed for 16 days, developed for 3 minutes in Kodak D-19 developer, fixed for 8 minutes in Kodak Rapid-fix, washed for 30 minutes, and stained with hematoxylin. Examination of the scored sites in the light microscope revealed a cluster of grains over and around a single mononuclear cell (Fig. 2). Cellular morphology was of moderate to poor quality because the preparations were not fixed at any time during the procedure; but it was possible to see that the elements which formed antibody were mononuclear and varied considerably in size. It was unlikely that the cells with grains over them were macrophages for two reasons: (i) Autoradiographic plaques appeared in x-ray and photographic films when thoracic duct cells were plated in agarose. There are few or no macrophages in rat thoracic duct lymph (*11*). (ii) The number of plaques ranged from zero to six when lymph node cells were first immunized with a heterologous protein, such as hen egg albumin, and when labeled BSA was later applied to the plated incubated cells. If macrophages were responsible for plaque formation, the number should have been equally as great as when the homologous antigen was used to immunize lymph nodes.

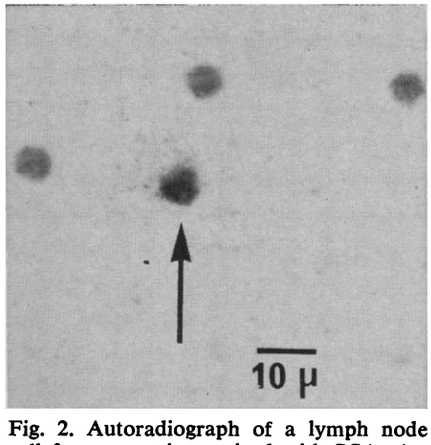

Fig. 2. Autoradiograph of a lymph node cell from a rat immunized with BSA. Arrow points to a cell covered by silver grains, presumably due to synthesis of γ-globulin which has bound specific antigen labeled with I^{131}. Three other cells without grains are present.

A roughly linear relationship between the number of autoradiographic plaques and the number of cells plated is indicated (Table 1). Immunized cells from both lymph nodes and thoracic duct lymph were capable of producing plaques.

The specificity of plaque formation was determined in several ways. Complete inhibition of plaque development could be accomplished by using unlabeled BSA. Slides were prepared with cells immunized with BSA and divided into two groups of 16 each. The first group was reacted for 24 hours with unlabeled BSA (1000 μg/ml), washed, and covered for an additional 24 hours with labeled BSA (1.0 μg/ml). The second group was reacted with labeled BSA only and yielded 540 plaques per 10^6 cells, or an average of 270 per slide. The inhibited group showed no autoradiographic plaques. Other controls for specificity included plating of nonimmunized cells from lymph node and thoracic duct lymph, plating of cells from lymph nodes regional to the injection of a noncross-reacting antigen such as hen egg albumin, and plating of immunized lymph node cells which were killed by heating to 56°C for 30 minutes. With these controls, between zero and six plaques per 10^6 cells were counted.

We are unable to state conclusively whether these "background" plaques represented an artifact of the method or whether they exhibited nonspecific binding of labeled antigen to precipitated rat γ-globulin. Eighty-five background plaques were sited by scoring the underside of slides of control preparations. The x-ray film was removed and replaced by NTB-2 track emulsion which was exposed for 16 days to the plated cells and then developed. In only one scored area we found a cell covered by grains similar to those seen over and around cells immunized with BSA. In 34 scored areas there was a focus of dirt or similar artifact in the agarose which could have been the cause of plaque formation in the x-ray film.

Two other complications were associated with the above method. The first was the appearance of pale autoradiographic plaques in the x-ray film. Such a result occurred most frequently when the specific activity of the labeled BSA was less than 1 μc per microgram. In some instances, pale plaques might have been the result of diminished synthesis of antibody by single cells. The second difficulty was the presence of high background, that is, excessive grains in the x-ray film, either focally or diffusely. High background was observed if the agarose was incompletely solubilized, if the surface of the plated slide was fingered, if labeled antigen was insufficiently washed from the preparations, or if a combination of these three situations occurred. To reduce high background, a protein, such as hen egg albumin, which is immunologically unrelated to BSA, was added to the solution containing labeled BSA in a concentration 50 times greater than that of the specific antigen. Despite these drawbacks, our method yielded a high proportion of usable slides, and the variation in number of autoradiographic plaques was no greater than the variation of hemolytic plaques observed in the Jerne technique.

This method for the development of autoradiographic plaques permits the use of a wide range of antigens, provided they can be labeled with a radioisotope. In addition, with the use of antiserums specific for the different classes of immunoglobulin, such as γG, γM, and γA, it aids the study of the kinetics and morphology of cells which synthesize these subgroups.

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8. Agarose was obtained from L'Industrie Biologique Française S. A.
9. The slide method and the Lucite racks were made available to us by Dr. R. I. Mishell.
10. Obtained from Eastman Kodak Company.
11. J. D. Feldman and R. Nordquist, in preparation.
12. Supported by NIH grant AI 7007. We thank P. Minden, S. Lee, R. Rickey, P. J. McConahey, and R. Nordquist for technical assistance. Publication No. 205.

17 March 1967

Penetration of an Organophosphorous Compound into Squid Axon and Its Effects on Metabolism and Function

Abstract. *The tertiary analogue of phospholine, namely,* $(C_2H_5O)_2P(O)SCH_2CH_2N(CH_3)_2$, *is a potent, irreversible inhibitor of cholinesterase which, when externally applied to the squid giant axon, readily penetrates in its inhibitory form into the axoplasm. However, even a* 10^{-2} *molar solution of this compound does not block axonal conduction unless the axon is first treated with a low concentration of venom from the cottonmouth moccasin. The question of the activity of acetylcholinesterase in these axons is considered, and the possibility of subcellular permeability barriers for individual components of the excitable membrane is discussed.*

The finding (*1*) of a high activity of an enzyme [phosphorylphosphatase (*2*)] that hydrolyzes diisopropylphosphorofluoridate (DFP) in parts of the squid giant axon has provided an explanation for the high external concentration of this compound required to block conduction. We have now used the dimethylaminoethylthio ester of diethylphosphoric acid, a powerful irreversible inhibitor of cholinesterase capable of crossing permeability barriers (*3*) and, in contrast to DFP, not subject to rapid enzymatic hydrolysis and detoxication. Although this and similar compounds are probably detoxified by microsomal enzymes (*4*), the rates of the detoxication are vastly slower than those for the enzymatic hydrolysis of DFP, sarin, tabun, and others of this acid anhydride group (*5*).

Dissection of the giant axon of the squid *Loligo pealii*, external recording of electrical activity, extrusion of axoplasm, and related techniques have been described previously (*6*). In deference to previous publications (*3*), we will refer to $(C_2H_5O)_2P(O)SCH_2CH_2N(CH_3)_2$, the tertiary analogue of phospholine, as 217AO (acid oxalate). The 217AO was dissolved in water; an amount, equivalent to that of 217AO, of calcium chloride in solution was added, and the *p*H was adjusted to about 7.5 with sodium hydroxide. The calcium oxalate was removed by centrifugation, and the supernatant was used after it was diluted to the desired concentration, tonicity, and buffer composition. For the determination of penetration, axoplasm was extruded from squid axons that had been bathed in a buffered sea-water solution of 217AO. This axoplasm, suitably diluted, was mixed with a sample of acetylcholinesterase (AChE) of known activity, and after 30 minutes the remaining AChE activity was measured manometrically. By comparison with a curve of the inhibition of AChE plotted as a function of known concentrations of 217AO, arithmetical corrections having been made for dilutions, the 217AQ concentration in the axoplasm, and hence its penetration, was determined. Using AChE from *Electrophorus electricus*, we estimated the pI_{50} of 217AO (negative log of the concentration of 217AO needed for 50 percent inhibition of AChE) to be 7.9. [The reported value for erythrocyte AChE is 7.9 (*7*).]

Table 1 shows that 217AO penetrates readily into the interior of the squid giant axon and even exceeds equivalent distribution in 1 to 2 hours. The methods and the calculations involved are exactly comparable to those reported previously, except that the ability to inhibit AChE was measured instead of C^{14} (*6*). Nachmansohn and associates (*8*) and Brzin *et al.* (*9*) examined the penetration of physostigmine, DFP, and paraoxon into the squid giant axon. However, paraoxon, which appeared to penetrate only a few percent, was probably mostly detoxified (*10*); the DFP was most certainly detoxified (*1*). Physostigmine, which obviously penetrated, is, like 217AO, rather poorly soluble in lipids. In addition, the inhibition of AChE caused by physostigmine is reversed fairly rapidly on removal of the inhibitor. We have attempted to avoid some of these difficulties by using 217AO, an irreversible and not readily detoxified inhibitor.

Table 2 shows the effects of 217AO on the electrical activity of the squid giant axon. In addition, although the effect of venom on the penetration of 217AO was not a part of this investigation, we did study the enhanced effects of 217AO on the action potential of squid axon after the axon had been treated with a concentration of cottonmouth moccasin venom (*Agkistrodon p. piscivorus*) which, of itself, had little or no effect on the action potential (*6*) (Table 2).

Inasmuch as Table 1 shows the penetration of 217AO in its active, inhibitory form, it may be concluded that detoxication is an insignificant factor in these experiments. However, the studies of penetration were performed with $10^{-3}M$ 217AO. Lest an enzyme system for detoxication be saturated at a far lower concentration and the percentage of detoxication, therefore, appear unusually small, we determined

Table 1. Penetration of $10^{-3}M$ 217AO, $(C_2H_5O)_2P(O)SCH_2CH_2N(CH_3)_2$, from a buffered sea-water medium into the axoplasm of squid giant axon. Temperature, 18°C; *p*H 7.8; results are shown as mean ± standard error.

Exposure time (min)	Experiments (No.)	Penetration (%)
5	2	49 ± 12
60	6	133 ± 21
120	4	147 ± 7

Table 2. Effect of 217AO on electrical activity of squid axons with and without previous treatment with venom. Those treated were incubated for 30 minutes in sea water containing 15 μg of cottonmouth venom per milliliter before exposure to 217AO. Results are shown as mean ± standard error.

Concentration of 217AO (mole/liter)	Experiments (No.)	Decrease of action potential (%)	Exposure time (min)
No previous treatment			
10^{-3}	8	6 ± 3	60
10^{-2}	4	15 ± 9	60
Previous treatment with venom			
10^{-3}	3	35 ± 8	30
10^{-2}	4	100 ± 0	5–30