Antibody Synthesis Initiated in vitro by Paired Explants of Spleen and Thymus

Abstract. *Primary in vitro synthesis of antibody has been achieved with a mouse spleen-thymus organ culture system 54 hours after it was incubated for 18 hours with coliphage R17.*

Many attempts have been made to develop an in vitro organ culture system in which antibodies could be synthesized in lymphoid tissue after incubation with antigen. Although considerable success in detecting antibody production has been achieved with culture systems utilizing organs previously immunized in vivo (*1*), the models developed for primary antibody synthesis have necessitated a preliminary step. For example, Fishman (*2*) and Friedman, Stavitsky, and Soloman (*3*) first incubate their antigen with large numbers of peritoneal macrophages. Globerson and Auerbach (*4*) inject either phytohemagglutinen or complete Freund's adjuvant intraperitoneally 24 hours before killing their animals for culture. The results from our experiments appear to indicate that a primary antibody response can be induced in vitro by a process more closely resembling the in vivo condition.

These experiments utilized spleen and thymus explants obtained from Swiss-Webster mice less than a week old, killed by decapitation. After death all animals were exsanguinated, the blood was collected and centrifuged, and the serums were saved for later titration. Explants (usually 7 to 8 in number) were placed in plastic filter-well assemblies resting in Falcon culture dishes 60 mm in diameter by 15 mm deep. The assembly consisted of a multichambered Teflon plate with 21 circular holes, each 5 mm in diameter and 5 mm in depth. A nylon-reinforced TW Millipore filter (pore size, 0.45 μ) was glued to the bottom of the Teflon plate.

Each experiment consisted of six filter-well assemblies, including two assemblies containing explants of spleen, two assemblies containing explants of thymus, and two assemblies containing explants of both spleen and thymus. The number of explants per culture dish varied with the age and size of the animals, but always included the entire spleen and/or thymus of each animal; tissues from different animals were never mixed. Where spleen and thymus were cultured separately, no more than one explant was placed in any one well of the assembly. Where spleen and thymus were cultured together, a single well contained an explant of each organ; contact between the spleen-thymus explants was not prevented. To each of the culture dishes, 3.0 ml of Eagle's basal medium (*5*) containing 10 percent calf serum was added, a volume sufficient to just cover the organ explants. One-half of the cultures served as controls; to the other half of the cultures a 0.1-ml volume containing 10^8 ultraviolet-inactivated plaque-forming units of the coliphage R17 was added as the antigen. The dishes were incubated in a water-saturated atmosphere containing 5 percent CO_2 and 95 percent air, at a temperature of 38.5°C.

After incubation for 18 to 24 hours the virus-treated explants were washed three times in Earle's balanced salt solution to remove excess antigen and were then placed in a new filter-well assembly with fresh medium; the controls were treated in a similar manner. The medium was changed and collected at 2, 5, and 8 days after washing. The used medium was stored at −20°C for later titration.

The globulin fractions contained in the harvested culture medium from each assembly were concentrated approximately six times. All operations for these procedures were carried out at 4°C. The globulin proteins were precipitated from the harvested medium by the addition of an equal volume of saturated ammonium sulfate. After low-speed centrifugation (1000*g* for 15 minutes) the packed precipitate was dissolved in 0.5 ml of 0.2*M* phosphate buffer (*p*H 7.6) (*6*). In order to remove the ammonium sulfate, the dissolved samples were dialyzed against 200 volumes of the phosphate buffer described above, changed three times at 12-hour intervals.

The concentrated fluids from cultures were titrated for R17-neutralizing activity as follows: To tubes containing duplicate samples of 0.2 ml of each fluid concentrate, 0.1 ml of a suspension containing approximately 100 active plaque-forming units of R17 was added. A series of control tubes containing 0.2 ml of saline and 0.1 ml of a suspension of R17 were also prepared. All tubes were incubated for an hour in a 37°C water bath. Immediately after incubation 0.1 ml of a suspension of the HFR1 strain of *Escherichia coli* (about 10^8 bacteria per milliliter) and 2.0 ml of melted 0.8-percent tryptone agar were added to each tube. The tubes were maintained in a 45°C water bath until they could be poured into petri dishes containing approximately 20 ml of a solidified 1.2-percent tryptone agar feeder layer. After solidification of the top agar the plates were incubated upside down overnight at 37°C. The next morning we counted and tabulated the number of plaques on each plate. The percent neutralization was calculated by comparing the number of plaques obtained on each experimental plate with the average number of plaques obtained from the series of virus-saline plates. For the purpose of these experiments a neutralization of 20 percent or more of the bacteriophage was considered significant. For a more extensive discussion of the agar overlay method of phage assay and titration, see Adams (*7*).

Table 1. Results of titration of tissue culture fluid concentrates collected 5 days after completion of incubation with antigen.

Filter-well assemblies (No.)	Type of culture in assembly	Inactivation (%)
	Unstimulated	
54	Spleen	0 to 10
50	Thymus	0 to 10
48	Spleen-thymus	0 to 10
	Stimulated	
54	Spleen	0 to 10
46	Thymus	0 to 10
32	Spleen-thymus	0 to 10
4	Spleen-thymus	30 to 40
6	Spleen-thymus	40 to 50
9	Spleen-thymus	50 to 60
3	Spleen-thymus	90 to 93

Neutralization of from 30 to 93 percent of the R17 plaque-forming units was observed in the concentrated fluids obtained from 22 of 54 stimulated spleen-thymus cultures. (Attempts are being made to increase the number of positive cultures, since at present they remain in the minority.) No significant neutralization (less than 10 percent) was observed in fluids obtained from any of the control cultures or from any of the stimulated but separately explanted spleen or thymus cultures (Table 1). In most experiments, neutralizing activity could

be demonstrated within 72 hours of antigenic stimulation, and was still detectable 10 days after stimulation, at which time the experiments were terminated. Both the number of successful experiments (22/54) and the inactivation titers obtained in our system appeared to be as high or higher than those obtained in other primary in vitro mammalian antibody systems in which bacteriophage has been the antigen (*2, 3*).

Preliminary experiments designed to show that the virus-neutralizing activity found in our concentrated culture fluids was due to an immunoglobulin and not to some nonspecific virus-neutralizing factor have been encouraging. The 7*S* and 19*S* gamma-globulin fractions were isolated from several of the concentrated culture fluids (6 days after antigen stimulation) with high inactivation titers by means of sucrose density gradient centrifugation. Titration of these and all other fractions of the density gradients showed the neutralizing activity to be concentrated only within the 7*S* and 19*S* peaks. Control samples showed less than 5 percent neutralization in all fractions. (Details of the above experiments as well as other experiments designed to characterize the nature of the neutralizing substance will be reported later.)

Assuming that the neutralizing activity directed against R17 demonstrated by the concentrated fluids was due to antibody, the possibility of a secondary response due to previous infection with this organism must be considered. Factors weighing against this possibility include both the early age of the animals at the time they were killed and the lack of any detectable neutralizing activity in their serums. However, since the R17 is a widely distributed coliphage, the possibility of primary in vivo exposure cannot be completely ruled out unless germ-free animals are used.

In order to detect the relatively small amounts of antibody produced in a system of this type, very sensitive assays are required. Two of the most sensitive systems include lysis of red blood cells as described by Jerne (*8*) and phage neutralization as described by Adams (*7*). Unfortunately, antigens found on red blood cells are as ubiquitous as are coliphages.

In the present experiments the presence of both spleen and thymus together in the cultures is required for production of the neutralization factor. The specific mechanism of spleen-thymus interaction in our system is yet to be defined. However, the lymphoid system of the mice used in these experiments is rapidly differentiating, and it has been widely reported that the thymus is required for the functional as well as morphological differentiation of lymphoid tissue (*9*). How the thymus functions in these respects is still obscure.

Especially significant in the above system is the absence of the requirement for any preliminary preparations of either antigen or tissues before primary stimulation with antigen.

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References and Notes

1. M. C. Michaelides and A. H. Coons, *J. Exp. Med.* **117**, 1035 (1963); G. S. Thorbecke and F. J. Keuning, *J. Immunol.* **70**, 129 (1953); A. B. Stavitsky, *ibid.* **75**, 214 (1955); S. H. Vaughn, A. H. Dutton, R. W. Dutton, M. George, R. Q. Marston, *ibid.* **84**, 258 (1960).
2. M. Fishman, *Nature* **183**, 1200 (1959); *J. Exp. Med.* **114**, 837 (1961).
3. H. P. Friedman, A. B. Stavitsky, J. M. Solomon, *Science* **149**, 1106 (1965).
4. A. Globerson and R. Auerbach, *ibid.* **149**, 991 (1955).
5. H. Eagle, *ibid.* **122**, 501 (1955).
6. S. P. Colowick and N. O. Kaplan, *Methods of Immunology* (Academic Press, New York, 1955), vol. 1, p. 143.
7. M. H. Adams, *Bacteriophages* [Interscience (Wiley), New York, 1959], pp. 450–451, 463–464.
8. N. K. Jerne and A. A. Nordin, *Science* **140**, 405 (1963).
9. R. Auerbach, *Nat. Can. Inst.* (monograph **11**, 23–33 (1963); O. Archer and S. C. Pierce, *Fed. Proc.* **20**, 26 (1961); J. F. A. P. Miller, *Lancet* **1961-II**, 748 (1961); ———, *Proc. Roy. Soc. London, Ser. B* **156**, 415 (1962); B. D. Jankovic, B. H. Waksman, B. G. Arnason, *J. Exp. Med.* **116**, 159 (1962).
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Radiocarbon Samples: Chemical Removal of Plant Contaminants

Abstract. *Roots and similar plant matter can be efficiently removed from charcoal samples by nitration and acetone leaching after preliminary removal of humic acids and lignin by standard procedures.*

Some samples on which radiocarbon dating is desirable are mixtures of finely divided charcoal, plant matter (mainly root fragments), and soil organic matter. Physical separation of the contaminating plant matter is quite impractical because of the small particle size and the similar bulk densities of the charcoal and root fragments. Such samples commonly have to be discarded as unsatisfactory for radiocarbon dating, yet the geochronological significance frequently makes it highly desirable to salvage the charcoal for dating. In addition, the requirements for purity become more critical as the age of the samples increases (*1*) and when the samples are to be isotopically enriched (*2*).

Three chemical methods for removing plant contaminants from charcoal that have been tested by treating samples made up entirely of modern grass roots are (i) "wet combustion" with perchloric and nitric acids, (ii) bleaching with sodium hypochlorite followed by digestion in 72 percent sulfuric acid, and (iii) nitration of cellulose and leaching with acetone after bleaching. The last method is the easiest and most efficient and is described in detail.

Washed and dried grass roots were leached with hot sodium hydroxide solution to remove humic acids and lignin. Final delignification was obtained by bleaching the residue with sodium hypochlorite in hydrochloric acid solution (*3*). The remaining cellulose residues were removed by nitration and subsequent extraction of the nitrated cellulose with acetone. The test sample of grass roots was rendered completely soluble by this treatment, the only residue being inorganic matter (probably silica).

To test the method further, an artificial sample was prepared by mixing 8.18 g of charcoal that had C^{14} activity (sample A-482) (*4*) with 0.47 g of modern (August 1963) grass roots (sample A-487) with a fallout-produced radiocarbon activity equivalent to 173±2.4 percent of the modern activity (0.95 of standard oxalic acid).

The artificial sample consisting of 94.61 percent charcoal and 5.39 percent dried roots was pulverized, boiled in 15 percent sodium hydroxide solution for 1 hour, and washed and filtered on glass paper. The residue was delignified by slowly adding 150 ml of sodium hypochlorite (Clorox) to the sample in 200 ml of 6*N* hydrochloric acid with constant stirring. After boiling this mixture for 20 minutes and filtering it, the cellulose in the residue was nitrated for 10 minutes with 400 ml of a mixture of concentrated nitric