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In vitro Production of Chicken Globulins and Precipitating Antibody*

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Summary. Immunofluorescent and isotope coprecipitation techniques provided methods of studying *in vitro* production of antibody by chicken spleen cells. In some culture preparations precipitating antibody was produced in sufficient quantity to be measured by quantitative techniques. The *in vitro* production of globulins by chicken spleen cells has been demonstrated by paper and immunoelectrophoresis of tissue culture medium. The antibody in the globulin produced in the *in vitro* tissue culture preparations may be shown by an autoradiograph of the immunoelectrophoresis after the addition of antigen trace labelled with radioactive iodine.

INTRODUCTION

Previous studies (Patterson, Suszko and Pruzansky, 1963) demonstrated that chicken spleen cells obtained after primary or secondary *in vivo* stimulation with heterologous albumins produced antibodies *in vitro* in tissue culture preparations. The antibody concentration in the tissue culture medium was determined by sensitive ^{131}I trace labelled antigen coprecipitation methods, and antibody producing cells were studied by parallel fluorescent antibody techniques (Patterson and Suszko, 1963). Primary and secondary responses of the spleen cells *in vitro* were consistent with established *in vivo* antibody responses of chickens.

The quantities of antibody produced by cells using *in vitro* tissue culture preparations reported by various investigators have been relatively small (Stavitsky, 1961). Various micromethods have been required for the detection of the antibody in the tissue culture medium. Using the methods described for the *in vitro* production of chicken antibody (Patterson *et al.*, 1963), it was observed that in certain cultures the chicken spleen cells produced sufficient antibody to be detected by ring precipitin techniques. This report describes the *in vitro* production of precipitating antibodies and the *in vitro* production of chicken globulins and their demonstration by electrophoretic and immunoelectrophoretic techniques.

MATERIALS AND METHODS

Immunization of Chickens and Preparation of Spleen Cells

Adult white Leghorn roosters received 50 mg. of horse serum albumin (HSA; Pentex Corp.) per kg. of body weight intravenously.

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Spleens were removed 5 days after antigenic stimulation for primary culture preparations and 4 days after secondary immunization for secondary culture preparations. The birds were bled for 40 ml. of blood and sacrificed. Spleens were removed and connective tissue was dissected away. The spleens were minced until the largest fragments were about 0·5 mm. in diameter and the fragments were washed five times in tissue culture medium. The final wash was used as 'zero time' (control) medium. The spleen fragments were placed in Petri dishes with cover glasses on the bottom of the dishes. Twelve dishes were used for each spleen, each dish receiving approximately the same amount of tissue. Tissue culture medium was Medium 199 (Hanks's base, pH 7·6, Cappel Laboratories) to which was added either sterile chicken serum or sterile chicken serum albumin (Pentex Corp.) to a final concentration of 15 per cent. One hundred μg. of streptomycin and 100 units of penicillin per ml. of medium were added. Cells from one Petri dish were removed, finely ground and extracted as a control ('initial extract') to determine the amount of antibody in cells at initiation of the culture.

In vitro secondary stimulation of antibody producing cells in tissue culture was carried out 3 weeks after *in vivo* stimulation. The spleen was removed and cells prepared in the usual manner. HSA was included in the tissue culture medium in a final concentration of 10 μg. N/ml. and used as the antigenic stimulus. After 3 days of incubation the medium with remaining HSA was removed and the cultured cells washed five times in fresh medium without HSA. Subsequent methods were identical with those of *in vivo* primary and secondary stimulation.

^{131}I Labelling of Antigen

HSA was labelled with ^{131}I (I*) using the method of Talmage, Baker and Akeson (1954).

Paper and Immunoelectrophoresis of Tissue Culture Medium

Paper electrophoresis was done on a Spinco paper electrophoresis apparatus. Immunoelectrophoresis was done on 5 × 15 cm. slides in 0·8 per cent Ionagar in pH 8·6 barbital buffer, ionic strength 0·075 for 12 hours at a constant current of 6 mA. per slide. Troughs were filled with pooled rabbit anti-normal chicken serum, and incubated at 4° for 1 week.

Autoradiographs of Immunoelectrophoresis Slides

Immunoelectrophoresis slides prepared as described above were washed for 24 hours in 0·15 M NaCl. The central trough was filled with I*HSA (10 μg. N/ml.) and incubated for 24 hours at 4°. The slides were washed in 0·15 M NaCl for 24 hours, dried with the gel in contact with filter paper and X-ray film exposed by contact with the dried gel.

Detection of Antibody in Tissue Culture Medium

Routinely, chicken anti-HSA in tissue culture medium was detected by a modification of an ammonium sulphate coprecipitation method (Farr, 1958) previously applied to detection of antibody in tissue culture preparations (Patterson *et al.*, 1963). Ring and gel diffusion precipitin reactions were done by standard techniques (Kabat and Mayer, 1961).

Medium containing sufficient precipitating antibody to measure quantitatively was studied by direct precipitation of I*HSA (Talmage and Maurer, 1953).

Fluorescent Antibody Studies of Tissue Culture Preparations

At each tissue culture medium change, cover slides were removed and cells stained for the presence of HSA or anti-HSA by the method previously described for immunofluorescent studies of tissue culture preparations (Patterson and Suszko, 1963).

RESULTS

PRODUCTION OF ANTIBODY *in vitro*

The results of nineteen successive cultures are shown in Table 1. The radioactive isotope coprecipitation test for antibody and immunofluorescent studies of antibody producing cells were most useful in detection of antibody. Both of these tests for antibody production *in vitro* were positive in successful cultures. The immunofluorescent evaluation

TABLE 1

RESULTS OF SUCCESSIVE TISSUE CULTURE PREPARATIONS OF CHICKEN SPLEEN CELLS

Culture	*Antigen stimulus*	*Tests for antibody*				*Notes*
		Immunofluorescent	*Isotope coprecipitation*	*Ring precipitin*	*Direct precipitation*	
A	Primary	+	+	−	−	
B	*in vivo*	+	+	−	−	
C		−	−	−	−	
D		+	+	+	−	
E		−	−	−	−	Mould contamination
F		+	+	+	+	Bacterial contamination
G		−	−	−	−	
H		+	+	+	−	
I	Secondary	+	+	−	−	
J	*in vivo*	+	+	−	−	
K		+	+	+	−	
L		+	+	−	−	
M		+	+	−	−	
N		+	+	−	−	
O		+	+	+	+	
P		+	+	+	+	
Q	Secondary	−	−	−	−	Mould contamination
R	*in vitro*	−	−	−	−	
S		+	+	−	−	

is the most sensitive because of the detection of antibody in single cells but has the disadvantage of not being quantitative as presently used. Five of the eight primary responses were successful after *in vivo* stimulation. All secondary responses after *in vivo* stimulation with antigen were successful in production of antibody *in vitro*, while one of three secondary responses after *in vitro* stimulation was positive. Occasional cultures were unsuccessful because of bacterial or mould contamination of the cultures. The quantity of antibody produced in successful cultures was measured at each medium change by the ammonium sulphate coprecipitation method. Fig. 1 shows the amount of I*HSA coprecipitated by chicken globulin in serial medium changes of a spleen cultured 4 days after a second *in vivo* stimulation with HSA. Peak antibody levels were detected 2 days after initiation of culture and antibody production was not detected at the 6-day medium change. The amount of antibody in extracts of cells at initiation of culture (initial extract) was significantly less than the antibody content of tissue culture medium after 2 days of successful growth.

Six of the nineteen successful cultures resulted in positive ring precipitin reactions against heterologous serum albumin (Table 1) but the amounts of precipitating antibody were insufficient to measure by quantitative macroprecipitation techniques except in the medium of three culture preparations. Precipitating antibody was detected 2 days after initiation of cultures. The double gel diffusion precipitin reaction of the medium from a secondary antibody response after *in vivo* antigenic stimulation is shown in Fig. 2. The

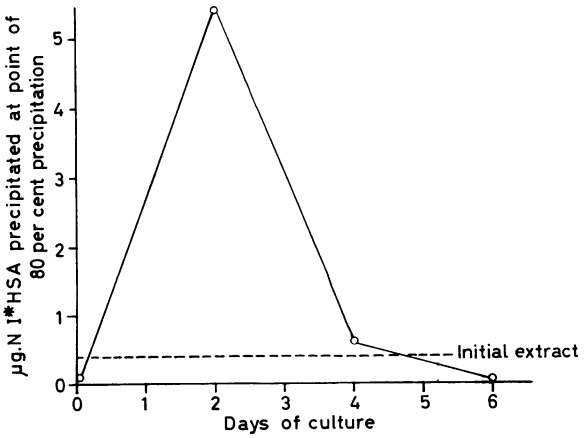

FIG. 1. Antibody content of tissue culture media demonstrated by ammonium sulphate coprecipitation of I*HSA by antibody in 1 ml. of medium obtained serially.

precipitating antibody in the medium producing this reaction could be measured by direct precipitation of I*HSA (Fig. 3). The usual type of chicken antibody curve (Patterson *et al.*, 1963) was obtained with the antibody present in the tissue culture medium. One ml. of tissue culture medium precipitated about 1 µg. of I*HSA N at maximum precipitation.

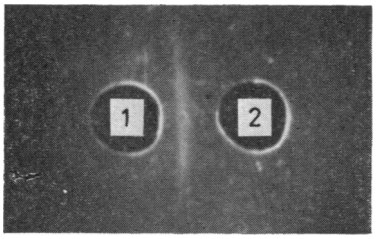

FIG. 2. Double gel diffusion precipitin reaction demonstrating anti-HSA produced in tissue culture. 1, Tissue culture medium after 48 hours of culture; 2, HSA.

ELECTROPHORETIC CHANGES IN TISSUE CULTURE MEDIUM

Tissue culture medium containing 15 per cent normal chicken serum obtained at zero time and 48 hours after initiation of culture was concentrated 5 : 1 by evaporation in the cold. Paper electrophoresis of the concentrates (Fig. 4a) showed a relative decrease in albumin and an increase in the proteins migrating as slow globulins. In order to clarify this change in electrophoretic pattern, chicken serum albumin was substituted for chicken

serum in tissue culture medium. Paper electrophoretic patterns of zero time and 48 hours medium showed a relative decrease in the albumin and increase in the slow migrating globulins (Fig. 4b). The decrease of albumin suggests catabolism of the homologous albumin in the tissue culture preparation.

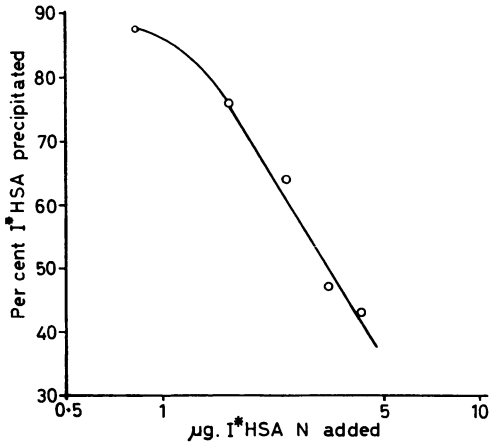

FIG. 3. Direct precipitation of I*HSA by 1 ml. of tissue culture medium after 48 hours of growth.

The same media which were subjected to paper electrophoresis (Fig. 4) were studied by immunoelectrophoresis to demonstrate the globulins produced *in vitro* in the tissue culture preparation, as seen in Fig. 5.

Immunoelectrophoresis of the zero time medium shows only an albumin band representing the chicken serum albumin used as nutrient in the medium. No globulins are

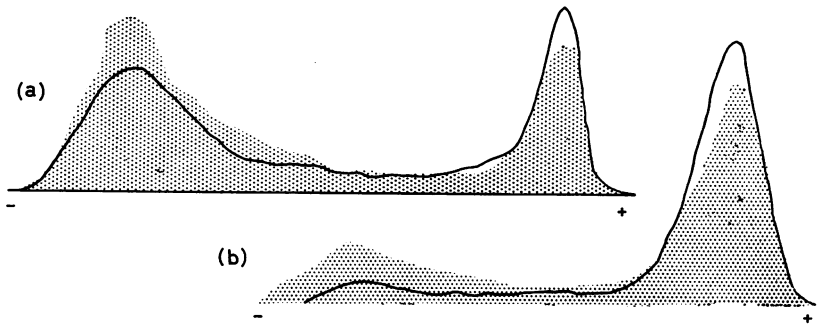

FIG. 4. Paper electrophoresis patterns of concentrated media from an antibody producing tissue culture. Dark line indicates pre-culture pattern and cross-hatching indicates pattern after 2 days of *in vitro* culture. Hanks's medium with 15 per cent normal chicken serum.

demonstrated in the zero time medium by this preparation. The medium obtained after 48 hours of culture has several bands migrating as globulins. Two of these bands are distinct. At least one additional band is faintly visible. These results demonstrate the formation of more than one globulin by the *in vitro* tissue culture preparation. I*HSA was added to the central trough of a preparation such as that shown in Fig. 5, incubated, washed, dried and used to prepare an autoradiograph with X-ray film. The binding of the I*HSA to the globulin bands (Fig. 6) demonstrated that the globulin bands shown in

Fig. 5 contain anti-HSA antibody. The preparations of autoradiographs of 48-hour culture medium indicated the presence of more than one precipitin band. This is shown

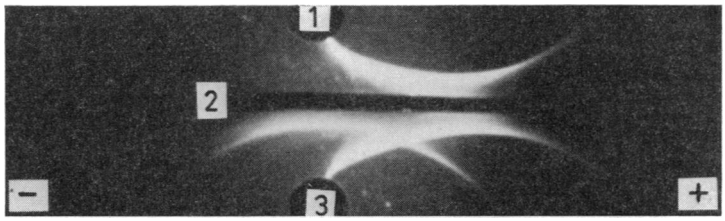

FIG. 5. Immunoelectrophoresis of media from the antibody producing tissue culture shown in Fig. 4(b). 1, Zero time medium; 2, rabbit anti-chicken serum; 3, 48-hour medium. Albumin band observed in the zero time medium with at least two additional slower migrating bands apparent after 48 hours of culture.

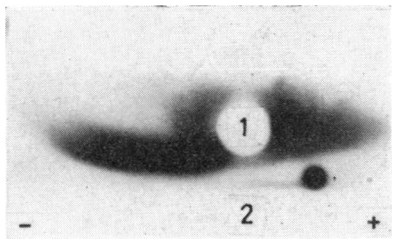

FIG. 6. An autoradiograph of immunoelectrophoresis of tissue culture medium containing antibody. 1, 48-hour medium. The trough (2) contains rabbit anti-chicken serum followed by I*HSA.

in Fig. 6 although reproduction of the autoradiographs by photography resulted in loss of definition of the bands.

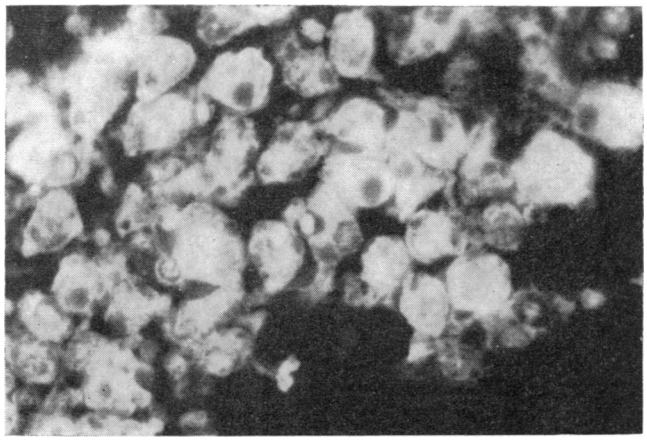

FIG. 7. Two-day culture preparation stained for anti-HSA antibody. Almost all cells are positive. ×2500.

FLUORESCENT ANTIBODY STUDIES OF TISSUE CULTURE PREPARATIONS

Staining of tissue culture preparations for antibody-containing cells provided supplementary information. While the immunofluorescent studies were qualitative, the appearance of a large number of antibody producing cells (Fig. 7) indicated a successful culture.

The method is highly sensitive in detection of small amounts of antibody and positive cells were detected when tissue culture medium contained only negligible amounts of antibody. Antibody-producing cells of different morphologic characteristics can be observed in the same and different preparations. Fig. 8 shows the plasma cell type seen predominantly in

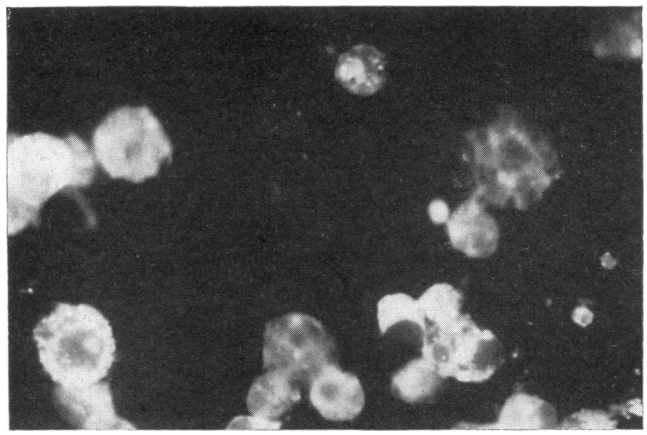

FIG. 8. Two-day culture preparation stained for antibody. A round cell in the lower left stains vividly for antibody. A plasma cell type is in the lower middle and a large round cell, probably a macrophage, present in the upper right. ×2500.

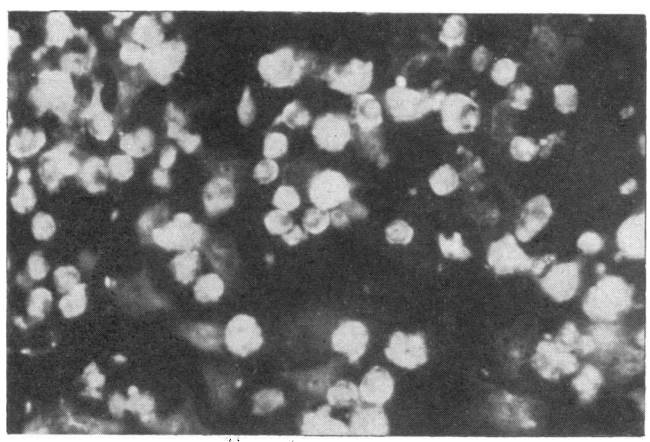

FIG. 9. Two-day culture preparation stained for antibody. Vividly positive cells contrast with negative cells faintly visible by autofluorescence. The latter have some fibroblastic characteristics. ×1200.

the secondary response, and also the large round cells with a rim of antibody containing cytoplasm seen in both the primary and secondary response. Fibroblastic proliferation (Fig. 9) in a culture was evidence of the growth of a culture and increasing number of these cells were seen in older cultures.

DISCUSSION

Successful tissue culture preparations using chicken spleen cells following *in vivo* primary and secondary stimulation have demonstrated antibody production *in vitro*. Both primary and secondary stimulation have resulted in production of precipitating antibody in detectable amounts in the tissue culture preparations. The chicken has been useful for study as a good *in vivo* producer of precipitating antibody after a single injection of a heterologous protein. The chicken spleen is an important site of antibody formation. These factors combine to result in the success of culture of chicken spleen cells for the production of precipitating antibody.

Variation in the total measurable antibody produced by different cultures has been observed. The fluorescent antibody technique is probably the most sensitive test of antibody production since it can demonstrate antibody in a single cell. The application of this technique to the study of antibody-producing cells in tissue culture appears particularly useful since there are both isolated cells and clusters of cells in the same preparation. Several methods of detection of antibody in tissue culture medium are available; the tanned red blood cell haemagglutination reaction is perhaps the most widely used. An isotope coprecipitation technique as used in the present studies is an additional useful method; either an ammonium sulphate coprecipitation or an antiglobulin coprecipitation test could be used for this type of study.

As various individuals of a species produce varying amounts of antibody following immunization, so the tissue culture preparations from the reticuloendothelial tissue of those individuals will produce different amounts of antibody. Variations in culture preparations may be greater because of the multiplicity of factors affecting growth encountered in tissue culture procedures. Those highly successful tissue culture preparations which produce precipitating antibody *in vitro* in amounts sufficient to be measured by quantitative techniques should provide a useful method of study of antibody production and protein synthesis. Just as variations in the quantity of antibody produced occur, so variations occur in the amount of globulins produced *in vitro*. Success with cultures can vary from slight antibody production demonstrated by moderate cell growth and positive staining for cellular antibody to highly successful preparations demonstrating precipitating antibody and globulin production (Figs. 3 and 4) *in vitro*. The presence of antibody in the globulins produced *in vitro* can be demonstrated by autoradiographs of the immunoelectrophoretic plates. The total amount of globulin formed in tissue culture is probably not specific antibody against the immunizing antigen, and immunoelectrophoretic plates suggested that at least three globulins were formed *in vitro*. Autoradiographic analysis (Fig. 4b) suggested the presence of more than one anti-HSA antibody in the tissue culture medium. This may be compatible with the two antibodies against heterologous protein antigens described in chicken antisera by Orlans (1962). The further characterization of both the specific antibody and globulins produced by *in vitro* tissue cultures are under further investigation.

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