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Long-Term Synthesis of Antibody *in vitro*.* (31957)

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Antibody synthesis at the cellular level is a relatively rapid process. Yet the full expression of the immune response in terms of participation of different cell types, accumulation and degradation of circulating antibodies, diversity of immunoglobulins and evocation of immunologic memory requires extended observations. All of these aspects can be readily followed in the intact host but heretofore they have not been scrutinized in organ culture. Some authors terminated their observations after 2 or 3 days(1-3). In the work of others the organ culture ceased to function after a relatively short period of time(4-6). Among the various tissues studied, the lymph node has proved to be the most useful organ for demonstrating *in vitro* induced secondary responses, whereas under similar conditions the spleen has been shown to produce antibodies less frequently, and to our knowledge there are no reports of the ability of thymic tissue to form antibodies *in vitro*. The present report describes a method which has permitted long-term antibody production in culture of fragments of lymph nodes and spleen and, in addition, has demonstrated immunologic activity on the part of thymic explants.

Materials and methods. Organ culture. The medium employed consisted of solution 199 (7) supplemented by 1 μg of hydrocortisone succinate. Penicillin (100 units/ml), streptomycin (50 μg/ml) and Fungizone (2 μg/ml) were added. In some experiments this medium was further supplemented with normal rabbit serum in a ratio of 25 parts of serum to 75 parts of medium. The organ cultures were prepared from tissues of rabbits previously immunized with bovine serum albumin (BSA). In most experiments an effort was made to obtain the tissue from animals whose antibodies had declined significantly from the initial high levels. Spleen, popliteal lymph nodes and thymus were diced into fragments measuring no more than 1-2 mm in the longest dimension. These were explanted onto discs

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of semi-solid agar prepared in the following way: 20 ml of melted Noble agar prepared at a concentration of 0.5% in solution 199 was poured on the surface of solid agar (1.5%) contained in Petri dishes with a diameter of 1500 mm. After allowing the overlay to gel, a punch with a 20 mm diameter was inserted through both layers and the double disc was lifted by means of a spatula. The soft agar layer, measuring approximately 3 mm in thickness, was then allowed to slide off the solid layer and was placed in 60 mm × 15 mm Petri dishes. Ten fragments of a given tissue with a combined weight of 10 to 15 mg were distributed on the surface of each disc which was surrounded, but not covered, by 2 ml of medium. The entire medium was harvested every 3 days so as to allow replenishment with fresh nutrient and to obtain samples for antibody assay. The samples were tested immediately or stored at −20°C.

Treatment of organ fragments in vitro. Some of the organ fragments were exposed to BSA for a period of 2 hours at 37°C under 4% CO_2 in air. Following this treatment they were washed 3 times with medium 199 and explanted to the agar disc. To study antibody production emanating solely from the primary *in vivo* immunization, organ cultures were prepared with corresponding fragments but without antigenic stimulation *in vitro*. Except for avoiding contact with BSA these fragments were handled in precisely the same way as the treated fragments.

Antibody determinations. Tannic acid treated sheep RBC were coated with bovine serum albumin (BSA) and used in a passive hemagglutination test according to Stavitsky's modification of the Boyden technique(8). Prior to testing the tissue culture fluids were absorbed with sheep RBC.

Determination of antibody class. Centrifugation studies were performed in a sucrose gradient (10% to 37%) at 100,000 × *g* for 16 hours in a Spinco model L centrifuge equipped with a SW 39 rotor. Gradients were formed by layering 0.6 ml of 7 sucrose solutions differing by 4.5% in concentration. The gradients were allowed to stand for 2 hours at 4°C until the interfaces had disappeared and 0.4 ml of undiluted culture fluid placed on top. Following centrifugation fractions were collected from the top of the tube with a syringe fitted with a blunt needle. Usually, 11 fractions containing 0.4 ml were taken. These were assayed for total anti-BSA activity and subjected to treatment with 2-mercaptoethanol (2-ME). This treatment was carried out by adding to each aliquot an equal volume of 0.2 M 2-ME. The mixtures were allowed to stand at 37°C for one hour and at 4°C overnight before assay.

Experimental. Duration of antibody synthesis. An initial experiment was performed in which the spleen and popliteal lymph nodes were removed 60 days after a single immunization of a rabbit with BSA. This animal had been immunized by injection of 12.5 mg of antigen in saline into each of the footpads. Some of the fragments were exposed to BSA (0.5 mg/ml) dissolved in solution 199 and others were kept as unexposed controls. At this time the serum antibody titer was 1:800 as compared with the peak titer of 1:6400 at 2 weeks after immunization. The results are given in Fig. 1. It can be seen that without *in vitro* stimulation the spleen produced no antibodies throughout the entire period of observation. However, following contact with BSA *in vitro,* antibody synthesis became manifest at 6 days reaching a peak at 12 days and remained at this level through 42 days. Thereafter, the antibody level decreased, but a titer of 1:32 was still present at 84 days. The popliteal lymph nodes produced antibody even in the absence of an *in vitro* stimulation as shown by the fairly consistent levels of antibody between days 12 and 60. A much more vigorous and considerably prolonged antibody synthesis took place when these lymph node fragments were restimulated *in vitro* prior to explantation. Peak titers are seen on days 12, 18 and 42, after which time there was a sharp decrease in the amount of antibody. Nevertheless, as late as 108 days the fluids from the stimulated lymph nodes still contained antibodies at a titer of 1:64.

The importance of a serum factor(s). Ambrose(9,10) described a technique for demonstrating antibody synthesis in lymph node fragments maintained in a medium free of serum but supplemented with hydrocortisone.

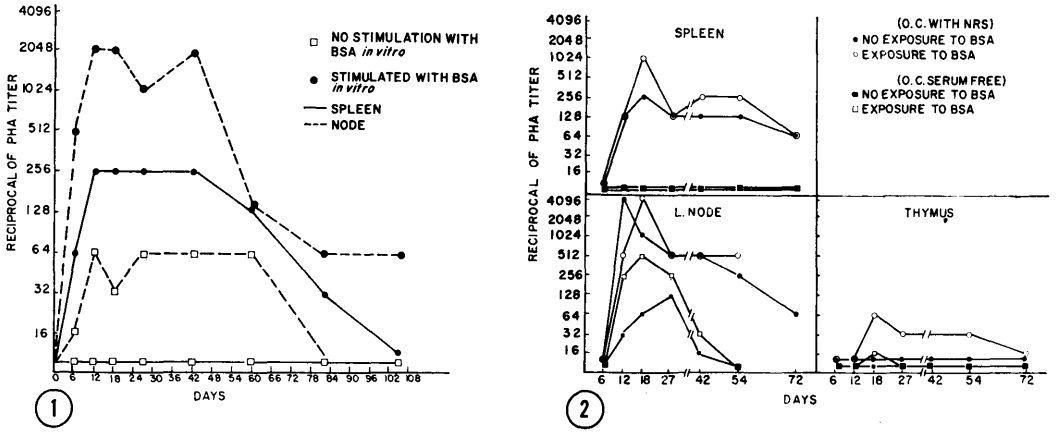

FIG. 1. *In vitro* antibody synthesis in spleen and lymph node of rabbit previous immunized *in vivo*. The secondary response following antigenic stimulation *in vitro* is pictured along with antibody production persisting from the primary immunization.

FIG. 2. Antibody production following secondary stimulation *in vitro* in spleen, lymph node and thymus. Organ cultures (O.C.) maintained in presence and absence of normal rabbit serum (NRS) are compared.

There is an obvious advantage in using a chemically defined medium not complicated by the addition of serum proteins. For this reason a series of experiments was performed in which spleen, lymph node and thymus, with and without *in vitro* antigenic stimulation, were cultivated in the presence and absence of normal rabbit serum. The results are given in Fig. 2. The lymph node fragments produced antibodies in the serum-free medium, but compared with the fragments in the complete medium antibody synthesis ceased much earlier. Moreover, the spleen and and thymus fragments were inactive in the serum-free medium but showed significant antibody production in the medium containing serum.

The antibodies produced *in vitro* were characterized in the ultracentrifuge using a sucrose gradient. In all samples of tissue culture taken on day 12 or later after explantation, peak antibody activity was located in the upper portion of the gradient in fractions 4 to 6. This antibody proved resistant to reduction by 2-ME. Similar results were obtained with subsequent harvests. Thus, all of the antibody detected *in vitro* had the character of 7S immunoglobulin.

Discussion. One of the basic questions raised in relation to tissue cultures pertains to the ability of the tissue and cell to retain its specialized function. The fact that lymphoid cells can continue to manufacture antibody *in vitro* is evidence that this kind of differentiation is retained *in vitro*. Moreover, since a secondary response can be elicited in such tissues *in vitro*, it must be assumed that the explants contain cells capable of recognizing the stimulus. In previous studies(5,10, 11) the duration of *in vitro* immunologic competency has been limited to a period of 26 to 34 days. The reason for the loss of activity is unclear. It is not known whether antibody production ceased because of loss of function on the part of the initially competent cells, loss of viability of the cells or the replacement of lymphoid elements by other cell types.

The present work describing a new procedure has extended the duration of immunologic activity. The data show that the cells of the lymph node retained their function for at least 108 days and the cells of the spleen for 84 days. In order to attain this prolonged activity, the tissues required the presence of serum. Ambrose succeeded in maintaining antibody production in lymph node fragments in serum-free medium supplemented by hydrocortisone but the duration of antibody synthesis in his medium was limited to about 20 days. Halliday and Garvey(12) while confirming the results of Ambrose with the lymph node failed to demonstrate antibody synthesis in spleen fragments even when these were de-

rived from rabbits hypermmunized intravenously. These findings are in accord with our data on the failure to demonstrate a synthesis in spleen fragments in serum-free medium. Kritzman and Harper(11) showed antibody production in spleen fragments extending up to a period of 34 days but, here again, the culture medium was reinforced by 20% serum.

The present system has also permitted the detection of antibody formation in organ cultures of fragments from the thymus. Thus far, antibody synthesis was noted only in those fragments which received a secondary stimulation *in vitro* as opposed to spleen and lymph node fragments in which, in addition to secondary responses, antibody production resulting from primary stimulation was still evident. The present findings cannot be construed as proof for antibody forming capability of thymocytes, as its quite likely that the antibodies were produced by lymphoid cells which may have been trapped in the thymus.

Explants on the agar surface gave rise to an outgrowth of cells which completely populated the surface of the agar. The nature of these cells is presently unknown although it should be stated that the methyl green-pyronin stain revealed cells with red cytoplasm. Morphologically, some of the cells resemble blast cells. Moreover, some of the cells continue to produce antibody after the original explants are moved to new Petri dishes. It should be noted that the transferred fragments also continued to produce antibody. We have not yet explored the state of cell division inside the fragments. There appears to be a loss of cells with time but it is unknown how much of the loss is due to migration and how much due to death of the cells. This type of study can best be done on lymph nodes which retain remarkably well their internal architecture in the present organ culture system.

The fact that the tissue fragments explanted on the agar surface retained their function for periods considerably longer than those reported in previous work implies that the culture substrate plays a significant role in the preservation of cell or tissue function. Wallace and Hanks(13) have previously demonstrated the retention of certain cellular functions (ciliary movement) of specialized cells explanted on the surface of agar. This work and the work of Gey(14,15) with collagen suggest that a gelatinous matrix favors the retention of cellular function. Heretofore, agar has been used principally as a substrate for the cultivation of bacteria and relatively little use of this gel has been made for cell culture, although agar overlays have been employed for the development of virus plaques in tissue culture monolayers. By virtue of its physical nature agar must exert a regulatory effect on the diffusion of nutrients and gases and the aggregation of cells thereby influencing the physiological activity of the cell with respect to growth, differentation and retention of function.

All antibody activity demonstrated in the organ cultures had the properties of 7S gamma globulin. While it is true that the passive hemagglutination test may have missed small amounts of 19S antibody, and more sensitive techniques, such as the antigen combining test, are in order, the fact remains that the system generated predominantly, if not exclusively, gamma G immunoglobulin. It should, therefore, prove useful as a source of one type of immunoglobulin and also as a model for morphologic and cytochemical studies on the synthesis of 7S immunoglobulin.

Summary. Organ cultures of spleen, lymph nodes and thymus, maintained on the surface of agar, were shown to retain the antibody-producing function for periods of time exceeding those previously reported. The long-term synthetic process requires the presence of normal serum. Such cultures continued to produce antibodies whose synthetic process was initiated *in vivo* and also manifested clear-cut secondary responses upon stimulation *in vitro*.

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Series Elasticity in Cat Papillary Muscle: Increased Stiffness After Segmental Damage. (31958)

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Hill(1-4) has described active muscle in terms of a mechanical model with 3 functionally separate components: (a) an active contractile element (CE) which is assumed to be freely extensible at rest, but which can shorten and develop force with activation, (b) a passive series elastic element (SE) arranged in series with the CE, and (c) a passive parallel elastic element (PE) which sustains resting tension. During isometric contraction, the activated CE shortens and stretches the SE with the development of force in the series elastic in accordance with its stress strain relations. The rate of force development (dP/dt) is thus directly proportional to the CE velocity (dl/dt) and the stiffness of the SE (dP/dl):

$$dP/dt = (dl/dt)_{CE} \cdot (dP/dl)_{SE}$$

The shortening of the CE is described by a characteristic hyperbolic relation between force and velocity(1,7), and the SE behaves like an exponential spring with an extension of 4-5% of initial muscle length (in heart muscle) during an isometric contraction(5).

The mechanics of normal heart muscle have been described in considerable detail(6-9) but there is little information available about muscle mechanics in myocardial damage. In particular, a reduction in the rate of tension development (dP/dt) in damaged muscle might be due either to a reduction in CE velocity (dl/dt) or to a more compliant SE (dP/dl). For example, ventricular aneurysm may be considered as a "series elastic disease" of the intact heart, wherein the effective SE of the myocardium is overly compliant due to ballooning of the aneurysm in systole, thus resulting in a diminished rate of tension development. The possibility that damaged heart muscle might also be overly compliant prompted the present study.

Methods. Each of 5 papillary muscles was rapidly removed from the right ventricles of cats anesthetized with intraperitoneal sodium pentobarbital (25 mg/kg), and was quickly suspended in a 20 cc bath filled with Kreb's bicarbonate solution at a constant temperature of 30°C and bubbled with 95% O_2 and 5% CO_2. Muscle lengths varied from 5 to 8 mm (excluding the tendon) with an average calculated cross-sectional area of .98 mm^2. The non-tendinous end was held by a spring clip extension of a force transducer, while the tendinous end was attached to the tip of a muscle lever(10) with a short length(3-4 cm) of 4-0 silk. The muscles were stimulated with platinum mass electrodes using pulses of 7 msec duration, voltages not more than 20% above threshold, and a stimulation rate of 12/min. Lever movement was detected by a photoelectric transducing system, and preload and afterload were added to a weight holder