A STUDY OF EXPERIMENTAL CONDITIONS AFFECTING THE SECONDARY IMMUNE RESPONSE IN VITRO

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INTRODUCTION AND REVIEW OF LITERATURE

That cells could be stimulated to produce antibody in tissue culture by direct addition of antigen was first demonstrated by Carrel and Ingebrigsten in 1912 (8). Many workers attempted unsuccessfully to stimulate antibody production in vitro using other antigens and tissues. Salle and McOmie (54) have reviewed the early work. Beard and Rous (4) attempted to stimulate Kupffer cells and clasmatocytes (macrophages) obtained from rabbits by incubating the cells with vaccinia virus. They were unable to detect antibody either in tissue culture or in the sera of rabbits into which the incubated cells had been injected intradermally. Selmar (56) tried to stimulate chick-embryo tissue with _Bacillus_ _abortus_ _Bang_ and reported negative results. Fastier (20), in an attempt to reproduce in tissue culture the series of cellular reactions which were thought to lead to antibody production in vivo, failed to stimulate rat macrophages, lymphocytes, bone-marrow, and spleen cells with suspensions of killed _Salmonella_ _paratyphi_ _B_. Roberts (50) incubated rabbit macrophages with _Salmonella_ _typhi_ in vitro but he too was unable to detect antibody formation.

A technique was reported by Harris and Harris in 1955 (27) which utilized normal rabbit lymph node and splenic cells incubated in vitro with *Shigella paradysenteriae*. Following incubation the cells were injected into normal rabbits that had been irradiated 24 hours earlier. Agglutinins were detected in the sera of the recipient rabbits on the 4th day after cell transfer. That a true antigenic stimulation in vitro of the lymph node cells had occurred and that the resulting agglutinin production was not due to active immunization by carry over of bacteria was demonstrated by Harris and co-workers (29, 31). Instead of whole bacteria as the antigen these workers incubated lymph node cells either with normal rabbit serum previously incubated with *Shigella* or with filtrates of trypsin-treated suspensions of *Shigella*. In both cases agglutinins appeared in the serum of the irradiated recipient rabbits on the 4th day after cell transfer. Similar results were obtained by Harris, Harris, and Farber (30) with blood leukocytes and with peritoneal exudate cells which were obtained 9 days after intraperitoneal irritation with lanolin plus light mineral oil. No agglutinins were detected in the recipient rabbits if the peritoneal cells were obtained 1 or 2 days after injection or obtained by injection of heavy mineral oil (30). A comprehensive review of this work has been prepared by Harris and Harris (28).

In 1957 a system of antigen stimulation and antibody formation in vitro was described by Kingsley, Stevens, and McKenna (37) and by Stevens and McKenna (62). These workers were able to induce a primary antibody response to bovine gamma-globulin in cultures of rabbit spleen cells if the doner rabbits had received 10 µgm. of purified endotixin from Salmonella typhosa 24 hours prior to removal of the spleen and addition of the antigen. Addition of antigen to untreated spleen cells did not initiate antibody formation. The effects of homologous and autologous sera and antigen concentration were also described (62). Utilizing the same system, McKenna and Stevens (41) later described work done with peritoneal exudate cells. Monocytes (the monocytes were separated from other peritoneal cells by their ability to attach to glass) which had been removed from peritoneal exudates of normal rabbits (untreated with endotoxin) 3 days after the injection of sterile mineral oil and exposed in vitro to either bovine gamma-globulin or egg albumin produced antibody for about 2 weeks, as assayed by the hemagglutination technique of Boyden as modified by McKenna (37). Treatment of the cells with endotoxin prior to addition of antigen made them respond as if they had been removed from hyperimmune rabbits. However, attention was called to the fact that the monocytes were induced by sterile mineral oil which may act differently from endotoxin but produce the same end result, i.e., ability to be stimulated by antigen in vitro.

Utilizing another approach to antigenic stimulation in vitro Michaelides (42) demonstrated a secondary antibody response of lymph node fragments removed from rabbits immunized with diphtheria toxoid. The tissue was maintained in vitro and hemagglutinating antibodies were detected on the 9th day in a small percentage of the cultures. Stavitsky (58, 59) using diphtheria toxoid as antigen and incorporation of radioactive amino acids for assay of the antibody, obtained similar results with both lymph node fragments and spleen fragments removed from immunized rabbits. Although the results obtained by the two workers were inconclusive, the evidence did suggest that a true secondary response was elicited in vitro.

The results of Michaelides (42) and Stavitsky (58, 59) opened a new approach for the study of antigenic stimulation and antibody production in vitro. That it was possible to initiate a secondary response in vitro was confirmed by Ambrose in 1962 (2) and by Michaelides and Coons in 1963 (43). Using fragment cultures of lymph nodes removed from immunized rabbits these workers consistently were able to initiate a secondary response in vitro to both diphtheria toxoid and bovine serum albumin. Antibody against diphtheria toxoid was synthesized for 4 days while synthesis of antibody against bovine serum albumin continued for 8 days. Experiments concerned with the conditions under which secondary antibody response could be obtained in tissue culture, the histological appearance of the tissue fragments, and minimal dosage of antigen required to obtain a detectable response were then described by O'Brien, Michaelides, and Coons (47). The effect of 5-bromodeoxyuridine was described by O'Brien and Coons (46). The results indicated that the response was dependent upon cell multiplication during the culture period. Ambrose and Coons (3) described the inhibitory effect of chloramphenicol on the synthesis of antibody in vitro. Their results indicated that chloroamphenicol inhibited some early phase of the response, possibly the messenger RNA of the stimulated cells.

Fishman (21, 22) reported another technique for primary antigenic stimulation in vitro. By incubating T2 bacteriophage or hemocyanin with rat macrophages and adding the cell-free filtrate derived from these macrophages to cultures of normal rat lymph node cells, specific antibody synthesis occurred. The active material in the cell-free filtrate was found to be RNase sensitive. It was essential that the antigen be incubated with the macrophages before addition to the lymph node cells for, as reported by Stevens and McKenna (62), direct addition of the antigen to the tissue culture failed to induce antibody formation. To further investigate the active material derived from the macrophages incubated with antigen, Fishman and Adler (23) used diffusion chambers charged either with the cell-free filtrate or with normal lymph node cells previously incubated with the macrophage material. The chambers were implanted in the peritoneal cavities of both irradiated and non-irradiated normal rats. Irradiated animals showed antibody formation if the chambers contained lymph node cells treated with RNA from macrophages incubated with T2 phage. If the chambers contained only the macrophage RNA, no antibody could be detected in these irradiated animals. In the non-irradiated rats, chambers containing only RNA initiated antibody production. Antibody was not formed by these animals if the macrophage material was treated with RNase. Friedman, Stavitsky, and Solomon (25) repeated the work of Fishman (21, 22) and confirmed and extended his results. They found by means of complement fixation studies that T2 head, tail, and internal protein antigens were associated with the RNA extracted from the macrophages previously incubated with the T2 phage. The importance of these contaminating antigens for antigenic stimulation of the nonimmune cells was discussed.

In studies paralleling those of Fishman (21, 22), Cohen and Parks (10) described the appearance of cells producing specific hemolysins in suspensions of nonimmune spleen cells after incubation with RNA extracted from the splenic cells of mice previously immunized with sheep erythrocytes. The number of hemolysin-producing cells was determined by the Jerne plaque technique (33, 34). Extending this work, Cohen, Newcomb, and Crosby (9) described experiments which indicated that the conversion of the nonimmune spleen cells to antibody-forming cells by RNA was partially strain specific. Sucrose gradient centrifugation showed most of the active material to be in the 8 to 12S fraction of the total cellular RNA. Similar results were obtained by Friedman (24) who utilized the same assay system although the stimulation reported was considerably higher than that reported by Cohen and Parks (10).

Recently, reports have appeared in the literature describing "true" primary antigenic stimulation in vitro. In work paralleling that of Kingsley, Stevens, and McKenna (37), Stevens and McKenna (62), and McKenna and Stevens (41), Globerson and Auerbach (26) have demonstrated primary immune reactions, initiated and maintained in vitro, of spleen explants from mice pretreated with either phytohemagglutinin or adjuvant. Following the addition of sheep erythrocytes to the explant cultures, specific hemolysins and agglutinins could be detected. The phytohemagglutinin and adjuvant did not appear to be involved in the immune response itself but served only to promote proliferation of the cultured lymphoid cells. Tao and Uhr (66) found that lymph node fragments removed from normal rabbits could be induced to synthesize both 19S and 7S specific antibody to the bacteriophage ϕX174. The difference between the primary and secondary responses to the phage induced in vitro were shown to be very similar to those observed in vivo. Saunders and King (55) demonstrated a primary immune response using paired explant cultures of spleen and thymus removed from 1-week-old mice. Addition of coliphage R17 to the culture resulted in production of specific neutralizing antibodies. Addition of the antigen to separate spleen and thymus cultures did not result in antibody production. The most significant finding in these reports is the absence of the requirement for pretreatment of either antigen or tissue before primary antigenic stimulation in vitro.

As indicated above, several workers found that direct addition of antigen to fragments of lymphoid tissue from immunized animals could initiate the synthesis of specific antibody. With organized tissue only the product of antigenic stimulation, antibody, can be studied. Richardson and Dutton (49) have found that suspensions of spleen cells from rabbits immunized with sheep erythrocytes could be stimulated to produce high numbers of antibody-synthesizing cells in vitro. The use of suspended cells and the Jerne plaque technique for the detection of single antibody-producing cells permitted preliminary quantitation of cellular response to antigen in vitro.

The Jerne plaque technique has provided a relatively simple, quantitative method by which individual antibody-producing cells can be identified in large cell populations. Since the technique was introduced, many workers have utilized it to study cellular response to antigen.

Study of antigenic stimulation and antibody formation in vitro has provided valuable information concerning the relationships of the two phenomena. However, little is known about the cellular events that intervene. Studies on cytodifferentiation indicate that two distinct types of cells respond to antigen. The first type, which arises during early development, has been called a "pluripotential cell," i.e., it is uncommitted as to its pathway of differentiation or antigen to which it will respond. The second type of cell arises as a result of previous antigenic stimulation. It appears to be restricted in response to antigen and differentiation pathway. Although the cellular transformations that follow antigenic stimulation and the cell(s) that synthesize antibody have been reasonably well established, the identity of the precursor cell(s), i.e., those cells which receive the initial stimulation, still remains unresolved.

The cellular events that occur during transformation and which lead ultimately to antibody production and release are still obscure. A marked increase in the number of mitoses in the spleen and lymph nodes following the injection of antigen have been demonstrated by Wissler _et al_. (71). The size of these organs may double within a few days. The importance of cell replication to antibody production is also indicated by the suppressive effects of x-radiation and DNA inhibitors. Capalbo _et al_. (7) have studied the mitotic rates of cells after antigen stimulation. Using tritiated thymidine as a tracer, their results showed that antigenically stimulated cells undergoing a secondary response had a generation time of 10 to 12 hours whereas non-stimulated cells divide every 24 hours. The generation time of secondarily stimulated cells has more recently been shown to be between 7 and 9 hours (1).

Using a unique experimental system of single-cell antibody assay combined with autoradiography, Nossal and Makela have proved that antibody-forming cells undergo rapid multiplication (39, 45). Rats which had received one antigenic stimulus but were not at the time synthesizing significant amounts of antibody were given a single brief pulse of ^{3}H-thymidine. This was rapidly incorporated into the nucleus of any cell synthesizing DNA at the time of the pulse. By autoradiography of cell smears from the popliteal lymph node, information on the type of proliferation of the originally labeled cells in the resting node was obtained. A second antigen injection after the isotope pulse then allowed the resultant change in the proliferative pattern of the originally labeled cells to be measured. The results can be summarized as follows: (1) Nearly all plasma cells formed during the secondary response were labeled indicating they were the result of recent mitotic divisions of the cells labeled before the secondary antigenic stimulation. (2) The greatly increased number of labeled cells indicated that rapid cell multiplication had occurred to give rise to such cells. (3) Fully differentiated antibody-forming plasma cells do not divide. However, such cells are not old cells. It was estimated that at the height of the secondary response, 4 to 5 days after the second antigen injection, virtually all antibody-forming cells are not more than 48 hours old, i.e., all have undergone four to eight mitoses during the response. By means of fluorescein-labeled antibody, Leduc et al. (38) have followed the cellular changes in the lymph node of a rabbit after the injection of antigen. They concluded that the only difference between the primary and secondary antibody response at the cellular level was that the latter involved a greatly increased number of cells.

It is also known that antigenic stimulation increases the DNA synthesis of immunocompetent cells. Dutton and co-workers (14, 15, 16, 17) have shown that the addition of antigen in vitro stimulates both DNA synthesis and cell division in spleen cell suspensions from rabbits immunized months earlier. The response is antigen specific and dependent on antigen concentration. It can be transfered to normal recipient cells by "primed" cells which have been briefly mixed with antigen. The relationship between antigen and increased DNA synthesis remains to be elucidated.

The advent of the Jerne plaque technique for detection of antibody-producing cells has provided the first means for quantitating cellular response itself. Many workers have used the Jerne method to study cellular response to antigen in vivo and, to a limited extent, in vitro. It may prove possible to quantitate antigenic stimulation of immunocompetent cells in vitro. In a preliminary study Richardson (48) has shown that spleen cell suspensions from immunized rabbits can be used to study factors that affect the secondary response. Optimum conditions are yet to be achieved. This study was undertaken to examine the effect of various experimental conditions on antigenic stimulation of the secondary response in vitro.

MATERIALS AND METHODS

Antigen. Sheep red blood cells (obtained from the Michigan Department of Health, Lansing, Michigan) were used as the antigen. Fresh cells were obtained each week. Before each use the cells were washed three times in 0.85% sterile saline. All dilutions of the packed cells were prepared in 0.85% sterile saline.

Animals and immunization procedure. Young white rabbits, weighing approximately 2 kgs. each, were given a single intravenous injection of 6×10^8 sheep red blood cells. One week later and for three successive weeks thereafter the animals received four intravenous injections weekly of 6×10^8 sheep red blood cells. Approximately 12 days after the final injection, blood was collected and the hemolysin titer of the serum determined for each rabbit.

Culture medium. A modified Eagle medium (hereafter referred to as Eagle medium) was used for culturing all tissue. The medium consisted of Eagle's basal salts (18) with the addition of Eagle MEM amino acids (19), Eagle MEM vitamins (19), glucose, and streptomycin (5 µg per ml). The amino acids and vitamins were purchased from Microbiological

Associates Inc., Bethesda, Maryland. Fresh normal rabbit serum was added to make a final concentration of 15%. Glutamine was not included in the culture medium. The same medium, without the 15% rabbit serum, was used for the preparation of Eagle agar (described below). For the agar distilled water was used in place of the rabbit serum.

Glutamine. Glutamine (Nutritional Biochemicals Corp., Cleveland, Ohio) was not used as a regular constituent of the Eagle medium but was added to various sets of tubes to determine its effect on the secondary stimulation in vitro. The glutamine was prepared in distilled water at 50X concentration and stored at -10 C until used. Concentrations varying from 0.4 mmole glutamine per 10^7 spleen cells to 4.0 mmole glutamine per 10^7 spleen cells were used.

Nonessential amino acids. Nonessential amino acids (19), purchased from Microbiological Associates Inc., Bethesda, Maryland, were added to various sets of culture tubes to determine their effect on the stimulation in vitro. The amino acids were stored at 4 C until used. A concentration of 0.1 mmole amino acids per 10^7 spleen cells was used.

Sodium pyruvate. Sodium pyruvate (19), purchased from Microbiological Associates Inc., Bethesda, Maryland, was added to various sets of culture tubes to determine its effect on the stimulation in vitro. The pyruvate was stored at 4 C until used. A concentration of 1.0 mmole pyruvate per 10^7 spleen cells was used.

Collection and preparation of tissue. Following immunization, at least 6 months was allowed to pass before the rabbits were used. Individual rabbits were then sacrificed by exsanguination and the spleen removed aseptically. The tissue culture was prepared immediately, essentially by the method described by Vaughan et al. (70). The spleen was minced with scissors and forced with a plunger from a 10 cc. syringe through a stainless steel plasma sieve. Small amounts (5 to 10 ml) of phosphate buffered saline, pH 7.2 to 7.4 (PBS) containing 1% normal rabbit serum were added to the tissue during the sieving process until the cell suspension totaled 30 to 35 ml. The cell suspension was decanted into a 25 x 150 mm screw cap tube and centrifuged at 600 rpm for 10 minutes in a Servall Angle Centrifuge, Model NSE. The supernatant was discarded and the washing procedure repeated two times. Following the final wash the cell pellet was resuspended in a small volume of Eagle medium containing 15% fresh normal rabbit serum and filtered through nylon gauze. (The fresh rabbit serum was collected from female Dutch Belted rabbits the day before the tissue was prepared.) Filtration of the cell suspension helped remove cell clumps, connective tissue, and fibrous material that sometimes formed during the preparation. After filtration a 1:10 dilution of the cell suspension was prepared in 0.1% crystal violet in 0.1 M citric acid and all nucleated cells counted on a hemocytometer. The remainder of the cell suspension was placed in a milk dilution bottle and gassed with 5% CO_2 in 95% O_2. Additional medium was then added to the cell suspension to give a final cell count of 2.4 to 2.8 x 10^7 spleen cells per milliliter of medium.

Secondary antigenic stimulation. For secondary antigenic stimulation in vitro 0.01 ml of appropriately diluted sheep red blood cells was added per 10^7 spleen cells. Concentrations ranging from 2.5 x 10^2 to 8 x 10^6 red blood cells per 10^7 spleen cells were used at various times.

Cell culture. Aliquots of 0.5 ml and 1.0 ml of the spleen cell suspension were cultured in individual 16 x 125 mm screw cap culture tubes. Each tube was gassed, using a plugged pipette, with 5% CO_2 in 95% O_2 and sealed. The tubes were incubated at 37C in an upright position. Two days after the cell culture was prepared an additional 0.5 ml of freshly prepared Eagle medium was added to each tube initially seeded with 0.5 ml of the spleen cell suspension. For tubes which contained 1.0 ml of the suspension, approximately 0.8 ml of the old medium was carefully aspirated off and replaced with fresh medium. The fresh medium was added to the tubes carefully so the cell pellet was not disrupted. The tubes were again gassed, sealed, and replaced at 37C for the remainder of the culture period. When the effects of different atmospheres on the cultured cells were compared, duplicate sets of the culture tubes were gassed with 5% CO_2 in 95% air as described.

Assay for antibody-producing cells. The Jerne plaque technique (33, 34), slightly modified, was used to determine the number of antibody-producing cells. Plastic, grided petri dishes, 100 x 15 mm, (Falcon, No. 3030) were used for plating the spleen cells. Each plate contained a bottom layer of 19 ml of 1.4% Difco agar in Eagle medium. Fresh plates were prepared before each experiment. After pouring, the plates were incubated with their covers off for 2 hours at 37 C to remove excess water. The plates were stored inverted at 4 C until used. About 12 hours before use, the required number of plates was wrapped in aluminum foil, kept inverted, and placed at room temperature. It was necessary to wrap the plates so they could come to room temperature slowly, thus eliminating bubbles which form in the agar.

The overlay agar consisted of 0.7% Difco agar in Eagle medium. The agar was prepared in 16 x 125 mm screw cap tubes at the same time as the plates. Each tube contained 1.0 ml of 1.4% Eagle agar and 1.0 ml of Eagle medium. The tubes were gassed with 5% CO_2 in 95% O_2, sealed, and stored at 4 C until used. At the time of use the tubes were placed in a boiling water bath for at least 30 minutes. The tubes were then transferred to a 46 C water bath. Immediately before plating, 0.1 ml containing 3 x 10^8 freshly washed sheep red blood cells was added to each tube and the contents mixed well. The spleen cells were then added either directly or indirectly.

Direct addition was accomplished by gently agitating the tissue culture tube to disperse the spleen cell pellet. An aliquot of the suspension was then added to the 0.7% overlay agar containing the sheep red blood cells.

Indirect plating of the spleen cells was carried out by adding 4 ml of sterile saline to the tissue culture tube and centrifuging the contents for 10 minutes at 1000 rpm in a Servall Angle Centrifuge, Model NSE. The supernatant was discarded, the cell pellet resuspended in the remaining liquid, and the tube placed in the 46 C water bath. Immediately the overlay agar containing the sheep red blood cells was poured into the tube containing the spleen cells.

Following direct or indirect addition of the spleen cells, the contents of the tube were mixed well and poured onto the 19 ml bottom layer of Eagle agar in the petri dishes. The overlay was quickly spread by rotating the plate to form a very thin layer in which the spleen cells and sheep red blood cells were dispersed. The newly poured agar layer was allowed to solidify for about 5 minutes. The plates were then incubated at 37 C for at least 8 hours, usually overnight, in desiccator jars in an atmosphere of 5% CO_2 in 95% O_2.

After incubation, 1.5 ml of a 1:5 dilution of guinea pig complement (dried guinea pig complement with 0.1% sodium azide as preservative, Hyland Laboratories, Los Angeles, California) was added to each plate and the plates incubated at 37 C for an additional 30 minutes. The complement was poured off and the plates stained with freshly prepared 2% benzidine (10 ml 2% benzidine in glacial acetic acid, 4 ml 10% H_2O_2, 86 ml cold distilled water). The plates were stained for 1 to 2 minutes, the benzidine poured off, and the plates flooded with PBS for about 5 minutes. A second wash of buffer was then added for about 15 minutes. The plates were inverted and allowed to dry. Staining of the plates served two purposes. The plaques stood out more distinctly against the blue background and staining allowed the plates to be stored at 4 C until counting was completed. Plaques were counted under 7X to 10X magnification.

Localized hemolysis in agar for hemolysin determination. "Spot lysis" on conventional Jerne agar plates was used to determine hemolysin titers. Plates and overlay agar were prepared as previously described except that no spleen cells were added. Dilutions of serum or cell culture supernatants were prepared in sterile saline. Quantities of 10 μl of each dilution were spotted on the plate, 16 spots per plate. The plates were incubated for at least 2 hours in desiccator jars in an atmosphere of 5% CO_2 in 95% O_2. Plates were developed by addition of 1.5 ml of 1:5 diluted guinea pig complement with 30 minutes incubation at 37 C. The complement was poured off and the plates stained with 2% benzidine. The plates were graded as to the amount of hemolysis of the sheep red blood cells produced by the diluted samples. A grading system of 4+ to - was used. A grade of 4+ was given to a spot which showed complete lysis of the red blood cells. Lesser amounts of lysis were graded 3, 2, 1, and ±. A grading of 2+ was used as the endpoint.

RESULTS

Culture conditions affecting the secondary antigenic stimulation in vitro of spleen cells from immunized rabbits were studied in this investigation. The objectives were to determine the effect of: (1) various numbers of sheep red blood cells (antigen); (2) 5% CO_2 in 95% air; (3) glutamine, nonessential amino acids, and sodium pyruvate added to the culture medium; and (4) specific anti-sheep erythrocyte serum added to the cell cultures.

White rabbits were hyperimmunized by a series of intravenous injections of 6×10^8 sheep red blood cells. At least 6 months after the last injection, individual rabbits were sacrificed and the spleen removed. A suspension of the spleen cells was prepared in Eagle medium at concentrations ranging from 2.4×10^7 to 2.8×10^7 per milliliter. The cells were cultured in individual 16 x 125 mm screw cap tubes at concentrations ranging from 1.2×10^7 to 1.4×10^7 cells per tube. Secondary antigenic stimulation in vitro was initiated by addition of 0.01 ml of diluted sheep red blood cells per 10^7 spleen cells. Numbers ranging from 2.5×10^2 to 2×10^6 red blood cells per 10^7 spleen cells were used. The cells were incubated at 37 C for 5 to 7 days in an atmosphere of 5% CO_2 in 95% O_2. Fresh Eagle medium was added to the cell cultures approximately 48 hours after preparation. Individual cultures were assayed for antibody-producing cells by the Jerne plaque technique.

Figure 1 illustrates a typical response obtained with spleen cells from an individual rabbit. No increase in the number of plaque-forming-cells (PFC) was seen during the first 2 or 3 days. Between the 4th and 6th day the response appeared to show an exponential increase to a maximum number of PFC followed by a rapid decline. An exponential response is consistent with the idea that cellular replication is intimately associated with both the primary and secondary response.

Antibody-forming cells were detected by their ability to produce plaques when plated in 0.7% Eagle agar containing 3×10^8 sheep red blood cells. Plaques were produced by diffusion of antibody from the individual spleen cells and hemolysis of the surrounding sheep red blood cells in the presence of complement. A typical plate containing the plaques is shown in Fig. 15. Figure 16 illustrates a single cell in the center of a plaque. The hemolytic plaques varied considerably in size, the smallest being less than 0.1 mm in diameter and the largest about 0.6 mm in diameter. Both large and small plaques were present on the same plate (Fig. 17). No difference was seen between plaques produced by cells plated at 0 hour and by cells plated after 90 to

140 hours of tissue culture. The shape and clarity of the plaques produced by cells after 90 to 140 hours of culture was the same as those produced by cells at 0 hour.

It was found during the experiments that pronounced variations in the number of PFC were sometimes obtained from replicate spleen cell cultures, Figs. 2 and 3. Each bar represents the number of PFC obtained from replicate tubes of spleen cells. The mean for each set of numbers is indicated. The variation in individual cultures is clearly seen. At least three tubes from each set were sampled when the cells were plated. Usually the number of PFC obtained from replicate cultures was very close with an occasional very high or very low count. The high counts may be due to an additional mitotic division of the antibody-forming cells in the particular culture. In a similar manner, the low counts may be due to a particular culture replicating at a slower rate. Since it is reasonably well established that a logarithmic increase in stimulated and antibody-producing cells occurs prior to and during active antibody synthesis, a geometrical mean of the PFC was used for calculation of the data.

Effect of antigen concentration on stimulation. The number of sheep red blood cells added per 10^7 spleen cells was found to be of primary importance for obtaining maximum stimulation. Figure 4 illustrates the results obtained with spleen cells from rabbit number 32 using 1.6 x 10^3 to 10^6 sheep erythrocytes per 10^7 spleen cells for stimulation. Cultures receiving 1.6 x 10^3 red blood cells showed maximum plaque formation, 4600 PFC per 10^7 spleen cells, at 93 hours. The peak was followed by a sharp decline in the number of PFC. This response was 40 hours earlier than that of the remaining cell cultures. Spleen cells receiving 6 x 10^4 sheep red blood cells responded more slowly but attained nearly the same number of PFC. As the number of red blood cells added was increased the number of PFC decreased. Cell cultures receiving 2.5 x 10^5 red blood cells produced 1900 plaques while cultures receiving 10^6 red blood cells produced only 130 plaques per 10^7 spleen cells. Cultures with no antigen produced a maximum of 40 plaques.

Figure 5 shows the results obtained with spleen cells from rabbit number 41. Antigen ranging from 2 x 10^4 to 2 x 10^6 red blood cells per 10^7 spleen cells were used to stimulate the cells. Although the curves differ considerably from those in Fig. 4, the final results correlate well. Cell cultures receiving the lowest number of red blood cells (2 x 10^4) produced the highest number of PFC. Spleen cells stimulated with 2 x 10^5 erythrocytes showed nearly the same plaque production. Cell cultures receiving 2 x 10^6 red blood cells showed a reduced stimulation as was seen in Fig. 4. The number of PFC decreased between 93 and 116 hours in cultures with 2 x 10^4 and 2 x 10^5 red blood cells. Usually a steady increase was obtained until maximum plaque production was achieved, after which the number of PFC decreased.

The results obtained with cells from rabbit number 42 are shown in Fig. 6. The curves obtained from cell cultures with 4 x 10^4 and 4 x 10^5 red blood cells paralleled one another very closely throughout the response. Each showed very nearly the same PFC production at maximum response. Cultures stimulated with 4 x 10^3 red blood cells had a lesser response which occurred 20 hours earlier than that of cell cultures receiving higher numbers of antigen.

The results obtained with spleen cells from rabbit number 43 are illustrated in Fig. 7. A very low number of red blood cells, 2.5 x 10^2 per 10^7 spleen cells, was used to stimulate one set of cultures. This resulted in a reduced number of PFC. Cell cultures with 5 x 10^3 and 10^5 red blood cells showed very nearly the same response.

Figure 8 illustrates the results obtained with spleen cells from rabbit number 45. Cultures stimulated with 10^4 red blood cells produced a maximum response of 3000 PFC per 10^7 spleen cells. Cell cultures stimulated with 10^3 red blood cells produced a maximum of 1100 plaques. The curves of these two antigen concentrations parallel one another throughout the response. Cultures with 10^5 red blood cells produced 400 plaques at 118 hours. This response was lower and 22 hours earlier than that produced by spleen cells stimulated with a lower number of red blood cells.

Figure 9 demonstrates the increase to maximum plaque production followed by a sharp decrease in PFC obtained with spleen cells from rabbit number 50. The cell cultures were stimulated by addition of 2×10^4 to 2×10^6 red blood cells per 10^7 spleen cells. Cultures receiving 2×10^4 red blood cells produced the highest response, 14,000 PFC, while those receiving 2×10^6 red blood cells showed reduced or inhibited plaque production. The maximum plaque production occurred at 118 hours.

Maximum cell stimulation was achieved when the number of red blood cells added to the cultures was between 10^3 and 4×10^5 per 10^7 spleen cells. No one antigen concentration consistently gave maximum stimulation of the spleen cells.

Addition of more than 10^6 red blood cells per 10^7 spleen cells showed inhibition of PFC (Figs. 4, 5, and 9). This inhibition may be the result of overloading the spleen cells with antigen. A similar failure to respond occurred when very low numbers of red blood cells were added to the cell cultures (Fig. 6). Presumably fewer spleen cells were stimulated because of the low number of red blood cells present.

The maximum number of PFC obtained from 12 rabbits, 11 of which responded well, varied from 160 to 21,050 (Table 1). The difference in the response may be due to the differences in the individual rabbits. The time between hyperimmunization and sacrifice was considered as having possible relevance to the stimulation in vitro. No correlation between the two could be found (Table 1). A possible relationship between the hemolysin present in the serum of individual rabbits following hyperimmunization and the amount of stimulation in vitro was also considered (Table 2). Again no correlation was evident.

Maximum plaque production was usually obtained between 90 and 140 hours. A peak response was never obtained before 90 hours. Occasionally the maximum response did not occur until the cells had been cultured 160 hours (Fig. 6). The low response of the cells to the antigen during the early phase of the cell culture may be responsible for the delay in maximum plaque production.

Following the peak, the number of PFC decreased rapidly as illustrated in Figs. 4 and 9. Presumably this was due to death or rapid degeneration of the cultured cells. Therefore, in many instances no cells were plated after the maximum response was obtained.

Of 12 rabbits sacrificed, one failed to respond or responded only slightly to antigen after 115 hours of culture. The results of this rabbit are not shown. At the

Table 1. Antigenic stimulation in vitro of cultured spleen cells from immunized rabbits under standard culture conditions.

Rabbit No.	Time After Immunization (months)	Antigen Concentration (RBC/10^7 spleen cells)	Number of Plaque Forming Cells at Day:							
			0	1	2	3	4	5	6	7
30	10	2.5×10^3	12				12	5,150		
32	12	1.6×10^3	11				6300	600		
33	12	10^5	60			2700	1280			
40	6	2×10^5	17			1370	1130	13,180		
41	6	2×10^4	17			20	118	98	1700	
42	8	4×10^4	32			2	15	1,500	2000	4700
43	9	10^5	8			1200	2200	2,400		
45	10	10^4	40				1200	1,550	2870	
46	11	10^4	5					21,050		
47	12	10^4	2				4950	2,671		
49	4	3×10^4	14			13	6	160		
50	7	2×10^4	29			2540	2930	14,000	590	

Table 2. Relation of maximum number of plaque forming cells to hyperimmunization titer of the rabbits.

Rabbit No.	Titer Following Immunization*	Maximum plaque Production per 10^7 spleen cells
32	2000	6,300
41	4000	1,700
42	4000	4,700
43	2000	2,400
45	2000	2,870
50	2000	14,000

*Reciprocal.

time of sacrifice this rabbit was suffering from a severe ear infection. This may account for the inability of the spleen cells to be stimulated. It is conceivable that the health of the animal was so poor that the spleen cells were unable to respond.

Detrimental manipulation of the spleen cells during preparation of the suspension and/or cultural conditions may account for the inability of the spleen cells to be stimulated. The presence of extraneous material in the cell cultures may also be related to the stimulation process. On several occasions there was considerable fibrin formation in the cell suspension after filtration through the nylon gauze. The fibrin could be seen in individual culture tubes and in some, but not all, the response of the cells was low. The spleen cells may phagocytize the fibrin material making it difficult or impossible for them to take up the red blood cell antigen. Alternatively, the fibrin material may not be taken up by the spleen cells but it may block or inhibit them from phagocytizing the antigen. Either or both may be responsible for the inability of some spleen cells to respond to the antigen.

Tissue culture conditions as related to culture media and glassware may be responsible for poor stimulation of the spleen cells. If essential reagents and materials in the culture medium had deteriorated, survival of the spleen cells would be limited. Clean glassware is essential for tissue culture and if the culture tubes were contaminated with detergent or other materials a poor response could result. Finally, leakage of the oxygen atmosphere from the culture tubes was detrimental to the spleen cells on several occasions.

Effect of 95% air on spleen cell stimulation. An atmosphere of 5% CO_2 in 95% O_2 was used throughout this investigation for culturing the spleen cells. Of the numerous reports appearing in the literature from workers studying antigenic stimulation and antibody production in vitro, there is little consistency with respect to the atmosphere used for culturing the various cells. Thus, it seemed pertinent to this study to determine the effect of 95% air on the stimulation of the spleen cells. Replicate cell cultures were prepared using the two atmospheres. Figure 10 shows the results obtained with cells from rabbit number 42 using 4 x 10^4 sheep red blood cells per 10^7 spleen cells for stimulation. The two curves obtained parallel one another very closely throughout the entire response. The cells incubated in the air atmosphere produced a maximum plaque number of 7200 per 10^7 spleen cells whereas those incubated in oxygen produced a maximum response of 4600 plaques per 10^7 spleen cells.

Figure 11 shows the results obtained with cells from rabbit number 43 using 10^5 sheep red blood cells per 10^7

spleen cells. Again the curves paralleled one another but less closely than for rabbit 42. Spleen cells cultured in the oxygen atmosphere produced 2300 plaques per 10^7 spleen cells and cells cultured in the air atmosphere produced a maximum of 600 plaques. These results were the opposite of those obtained from rabbit number 42.

Figure 12 illustrates the results with spleen cells from rabbit number 45. Two antigen concentrations, 10^4 and 10^5 erythrocytes per 10^7 spleen cells, were used for stimulation. The differences in the response were less defined than those from the previous two rabbits. Spleen cells stimulated with 10^4 sheep red blood cells and cultured in an oxygen atmosphere produced a higher response (2900 PFC per 10^7 spleen cells) than did the cells receiving the same number of red blood cells but cultured in air (1700 PFC per 10^7 spleen cells). Spleen cell cultures stimulated with 10^5 red blood cells and incubated in an air atmosphere showed a higher response than did those cultured in oxygen stimulated with the same number of red blood cells. The cells cultured in air produced a maximum of 850 PFC per 10^7 spleen cells whereas those cultured in oxygen produced only 370 PFC per 10^7 spleen cells.

Although no consistent effect of atmosphere on the number of antibody-producing cells was obtained, differences were noted in the appearance of plaques produced by the cells. The spleen cells cultured in 95% oxygen consistently produced more distinct hemolytic plaques than cells cultured in air. This may indicate that the amount of antibody produced by the stimulated cells is in some way affected by the atmosphere in which they are cultured.

Effect of additives to the cell culture medium. In 1955 Eagle (18) demonstrated that glutamine was an essential amino acid for the propagation of mammalian cell cultures. In 1959 (19) he further demonstrated the requirement of exogeneous pyruvate and certain "nonessential amino acids" for growth of particular cells. Table 3 shows the results obtained with cells from three rabbits after the addition of glutamine (2 mmole per 10^7 spleen cells), nonessential amino acids (0.1 mmole per 10^7 spleen cells), and sodium pyruvate (1.0 mmole per 10^7 spleen cells) to the culture medium. The media additives were added to the cell cultures at 0 hour. All cultures showed enhanced PFC production in the presence of the additives. Spleen cells from rabbit number 32 showed the greatest increase in plaque production. Cultures stimulated with 2 x 10^5 red blood cells and containing all three additives produced nearly 37 times more plaques than did the cultures containing only antigen. In some instances the high number of PFC from cell cultures with no antigen and cultures with only the three additives made the response difficult to interpret as with cells from rabbit number 33.

Table 3. Effect of sodium pyruvate, nonessential amino acids, and glutamine on the number of plaque-forming cells produced by spleen cell cultures.

Rabbit No.	No. red blood cells per 10^7 spleen cells	Media additives in cell culture	Hour of plaque production	Mean No. PFC* per 10^7 spleen cells
30	10^6	-	117	842
	none	-	117	69
	10^6	+	117	1,002
	none	+	92	50
32	2×10^5	-	115	311
	none	-	115	25
	2×10^5	+	115	11,500
	none	+	115	400
33	10^5	-	88	1,270
	none	-	88	1,120
	10^5	+	88	3,520
	none	+	88	980

*Plaque-forming cells.

Glutamine was added in various concentrations to cell cultures to determine its effect on antigenic stimulation in vitro. Table 4 shows the results obtained with spleen cells from six different rabbits. Cell cultures from rabbit number 32 with 2 mmole of glutamine and 2 x 10^5 sheep red blood cells per 10^7 spleen cells produced more PFC at 115 hours than did cultures without glutamine. The maximum response from cell cultures without glutamine occurred at 142 hours and was higher than that from cell cultures with antigen plus glutamine. A similar response was obtained with spleen cells from rabbit number 45. In addition, when glutamine was added 48 hours after the cell culture was prepared only ½ as many plaques were produced as from cultures with glutamine at 0 hour. Inhibition of PFC by glutamine was seen with spleen cell cultures from rabbit number 33. The time of the maximum response was the same for cultures with and without glutamine. However, the high number of PFC in the cultures with no antigen with and without glutamine makes the significance of these results uncertain.

In contrast to these results, spleen cell cultures from rabbits number 43 and 47 with antigen plus glutamine produced more PFC than did cultures without glutamine. Cell cultures from rabbit number 43 stimulated with 10^5 red blood cells and with 2 mmole of glutamine added produced nearly 3 times the number of plaques than did the same cultures without glutamine. Two concentrations of glutamine were used in

Table 4. Effect of concentration of glutamine on the number of plaque-forming cells produced by spleen cell cultures.

Rabbit No.	No. red blood cells per 10^7 spleen cells	Glutamine concentration (m mole per ml)	Hour of response	Mean No. PFC* per 10^7 spleen cells
32	2×10^5	-	115	311
	none	-	115	25
	2×10^5	2.0	115	430
	none	2.0	115	400
33	10^5	-	88	1,270
	none	-	88	1,120
	10^5	2.0	88	820
	none	2.0	88	100
43	10^5	-	118	2,400
	none	-	118	48
	10^5	2.0	118	6,320
	none	2.0	118	50
45	10^5	-	118	364
	none	-	118	36
	10^5	2.0	118	880
	10^5	2.0 + 48 hrs.	118	480
	none	2.0	118	40

46	10^4	-	115	21,050
	none	-	115	30
	10^4	2.0	115	16,150
	none	2.0	115	290
	10^4	4.0	115	20,320
	none	4.0	115	30
47	10^4	-	93	4,950
	none	-	93	16
	10^4	2.0	93	16,470
	none	2.0	93	14
	10^4	0.4	93	10,200
	none	0.4	93	35

*Plaque-forming cells.

cell cultures from rabbit number 47. Those cultures with 10^4 red blood cells and 2 m mole of glutamine per 10^7 spleen cells produced 3 times as many plaques as did non-glutamine-containing cultures. Those cultures containing 0.4 mmole of glutamine produced twice as many plaques.

Somewhat different results were obtained with cell cultures from rabbit number 46. Cultures stimulated with 10^4 red blood cells and with 2 mmole of glutamine produced fewer PFC than did cultures containing no glutamine. When twice the concentration of glutamine was added, the plaque production was nearly equal to that of the cell cultures with only antigen.

The effect of glutamine on the stimulated spleen cells was inconsistent. Some cell cultures with glutamine responded early to the antigenic stimulation, but the response was well below that of cultures without glutamine. The presence of glutamine in a few cultures enhanced the production of PFC. In these particular cell cultures, the concentration of glutamine seemed to influence the number of PFC produced.

Effect of specific antiserum in the culture medium. Specific antiserum has been shown to inhibit antibody snythesis in vivo when administered before or shortly after antigen injection. To determine its effect on antigenic stimulation in vitro, specific anti-sheep erythrocyte serum with

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a 2+ "spot lysis" reading at a dilution of 1:2000 was added in varying concentrations to spleen cell cultures. Figure 13 illustrates the results obtained by adding 0.1 ml of undiluted and diluted antiserum to spleen cell cultures from rabbit number 43. All cultures were stimulated with 10^5 sheep red blood cells per 10^7 spleen cells. Specific antiserum was added at 0 hour. Bar I shows the plaque formation of cell cultures which received no antiserum. The maximum response of these cultures was 2200 PFC per 10^7 spleen cells. This response was used as a reference for the antiserum-treated cultures.

Bar II represents the plaque formation of cultures with 0.1 ml of undiluted antiserum. These cultures produced a maximum of 800 PFC per 10^7 spleen cells, 1400 fewer than the untreated cultures.

The response of cell cultures with 0.1 ml of a 1:10 dilution of antiserum is shown by bar III. A maximum of 1500 PFC per 10^7 spleen cells was produced by these cultures. This response was about twice that of cultures with undiluted antiserum.

Plaque formation of cultures with 0.1 ml of a 1:100 dilution of the antiserum is shown by bar IV. A maximum response of 1730 PFC per 10^7 spleen cells was obtained, slightly lower than the 2200 PFC produced from the untreated cultures. Bar V illustrates the number of PFC in cultures with no antigen.

Figure 14 illustrates the results obtained with spleen cells from rabbit number 45 using the same antiserum. The cell cultures were stimulated with 10^5 red blood cells per 10^7 spleen cells. The response of cultures which received no antiserum is shown by bar I. A total of 3800 PFC per 10^7 spleen cells was produced by these cultures.

Bar II illustrates the response of similar cultures which received 0.1 ml of undiluted antiserum at 48 hours. A maximum of 130 PFC per 10^7 spleen cells was obtained from these cultures, less than 4% of the response from untreated cultures.

The response of cultures with 0.2 ml of undiluted antiserum at 48 hours is represented by bar III. A total of 30 PFC per 10^7 spleen cells was produced by these cultures. The response was equal to that of the cell cultures with no antigen represented by bar IV.

The results obtained with cells from rabbits number 43 and 45 indicated that antigenic stimulation in vitro is inhibited by specific antiserum. The extent of inhibition appears to be related to the concentration of antiserum added to the cell cultures. The results with cells from one rabbit, number 45, indicate that the inhibition is more pronounced when the antiserum is added after 48 hours of culture rather than at 0 hour.

Specificity of antibody produced in vitro. To determine if specific antibody synthesis was occurring in vitro, the antibody released by the spleen cells was tested against red blood cells of five species. Cell cultures which had been maintained for 7 days were centrifuged at 3000 rpm for 30 minutes. The supernatant from replicate cultures was pooled and stored at -10 C until used. Conventional Jerne agar plates were prepared, each with red blood cells of a different species incorporated into the overlay agar. These plates were spotted with 10 µl of dilutions of the cell-free medium. After 2 hours of incubation at 37 C the plates were developed by addition of 1.5 ml of 1:5 diluted guinea pig complement and 30 minutes of additional incubation at 37 C. The plates were then stained with 2% benzidine. The results obtained from five individual spleen cell cultures from three separate rabbits are shown in Table 5. All of the cell cultures tested contained specific hemolytic antibody for sheep red blood cells. A weak reaction was obtained from three cell cultures with chicken red blood cells. The reaction may be due to antigenic determinants common to both the sheep and chicken erythrocytes.

Table 5. Specificity of antibody synthesized by spleen cells in vitro as determined by "spot lysis" of red blood cells of five species.

Rabbit No.	No. red blood cells per 10^7 spleen cells	Dilution of 7 day Culture medium	Red Blood Cell Specie				
			Sheep	Bovine	Horse	Chicken	Human
32	1.6×10^3	1:25	3	-	-	-	-
		50	2+	-	-	-	-
		100	2	-	-	-	-
		150	±	-	-	1	-
	2.5×10^5 plus glutamine, pyruvate, and nonessential amino acids	1:25	3+	-	-	-	-
		50	3	-	-	-	-
		100	2	-	-	-	-
		150	1+	-	-	-	-
40	2×10^5	1:25	3+	-	-	1+	-
		50	3	-	-	1-	-
		100	2+	-	-	-	-
		150	2	-	-	-	-
43	10^5	1:25	3+	-	-	1-	-
		50	3	-	-	-	-
		100	2	-	-	-	-
		150	1+	-	-	-	-
	10^5 plus glutamine	1:25	4-	-	-	-	-
		50	3+	-	-	-	-
		100	2	-	-	-	-
		150	1+	-	-	-	-

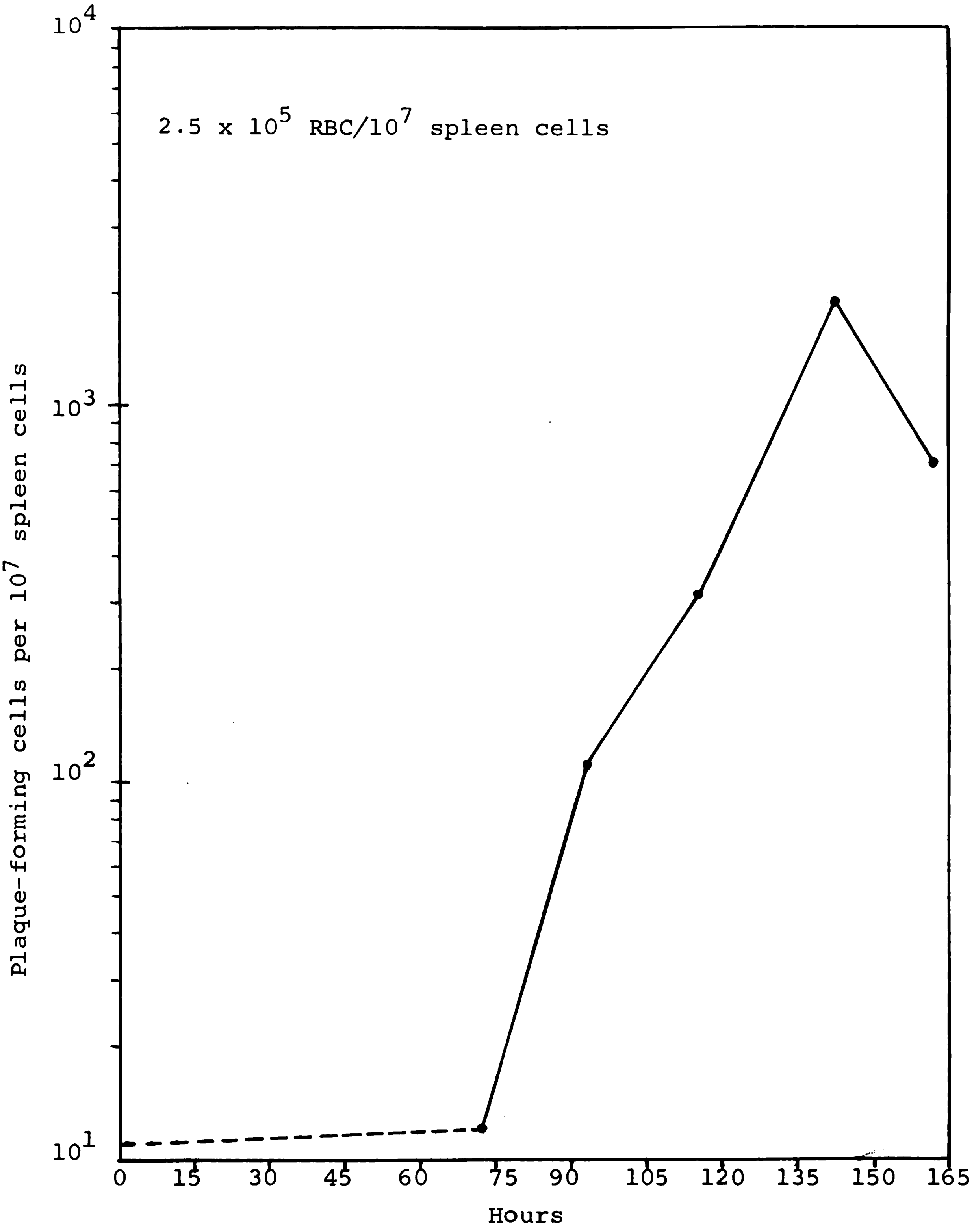

Figure 1. Response of spleen cells from rabbit number 32 to sheep red blood cell antigen.

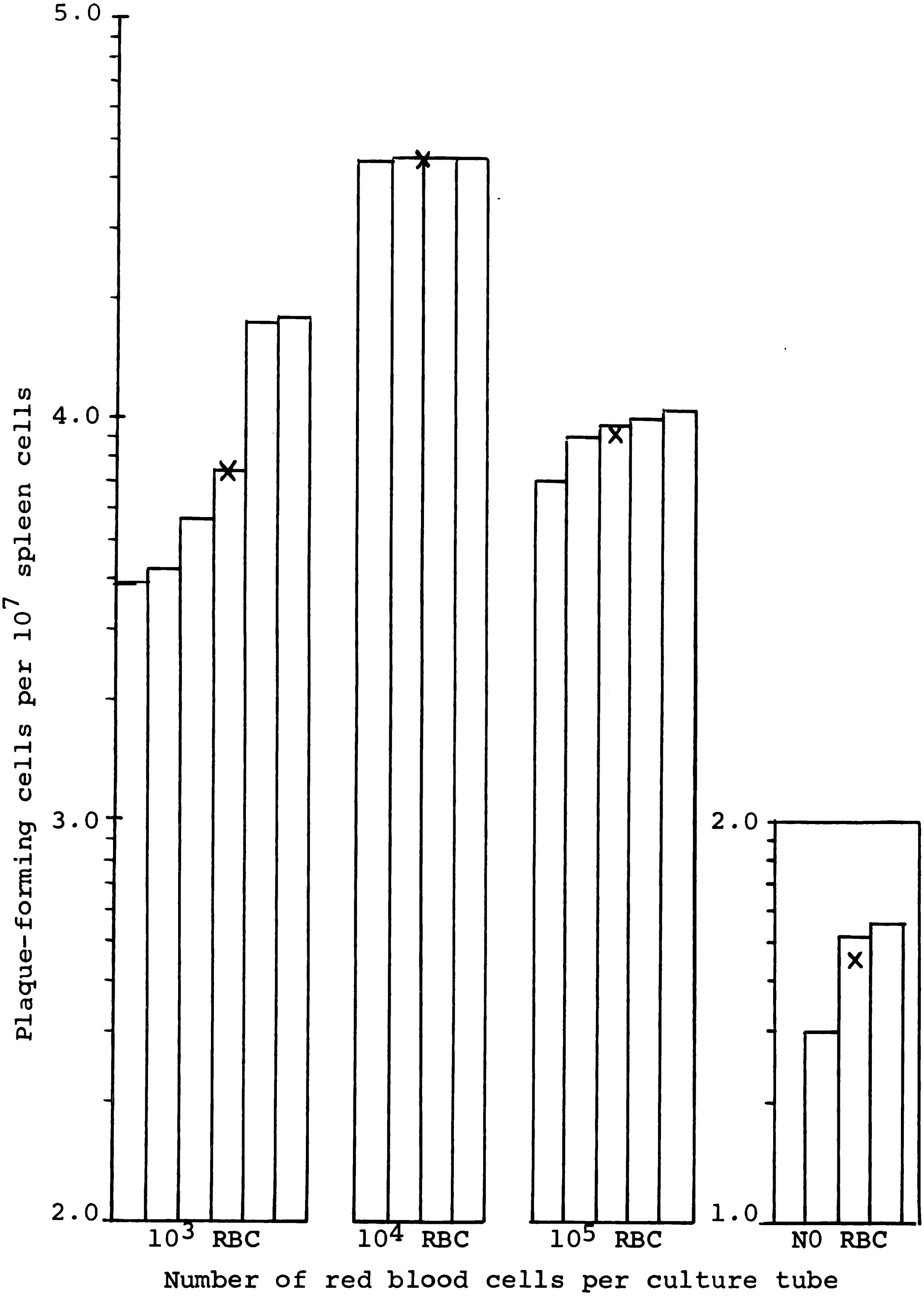

Figure 2. Logarithmic variation inthe number of plaque-forming cells produced by individual spleen cell cultures from rabbit number 46 after 115 hours of culture.

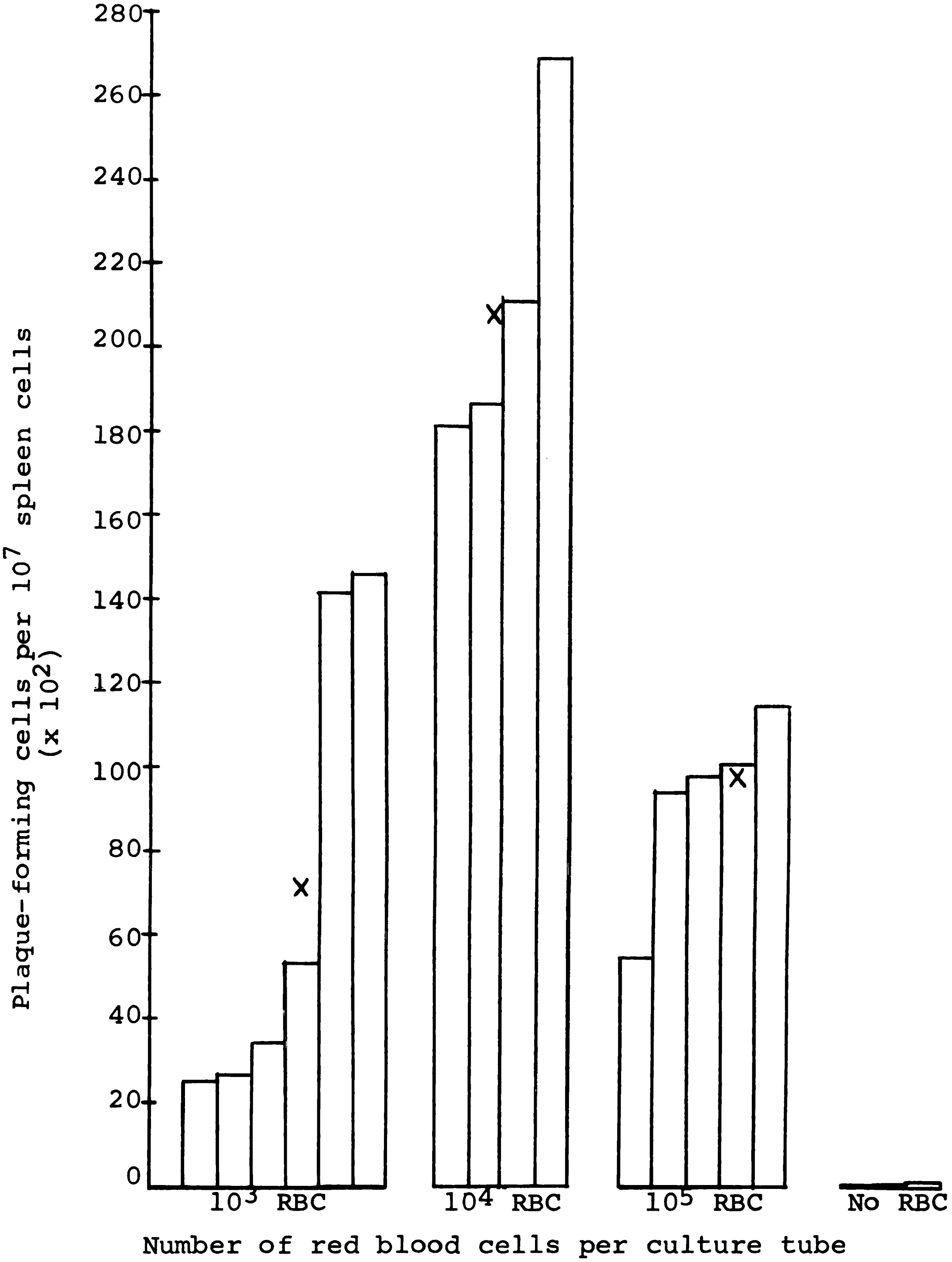

Figure 3. Arithmetic variation in the number of plaque-forming cells produced by individual spleen cell cultures from rabbit number 46 after 115 hours of culture.

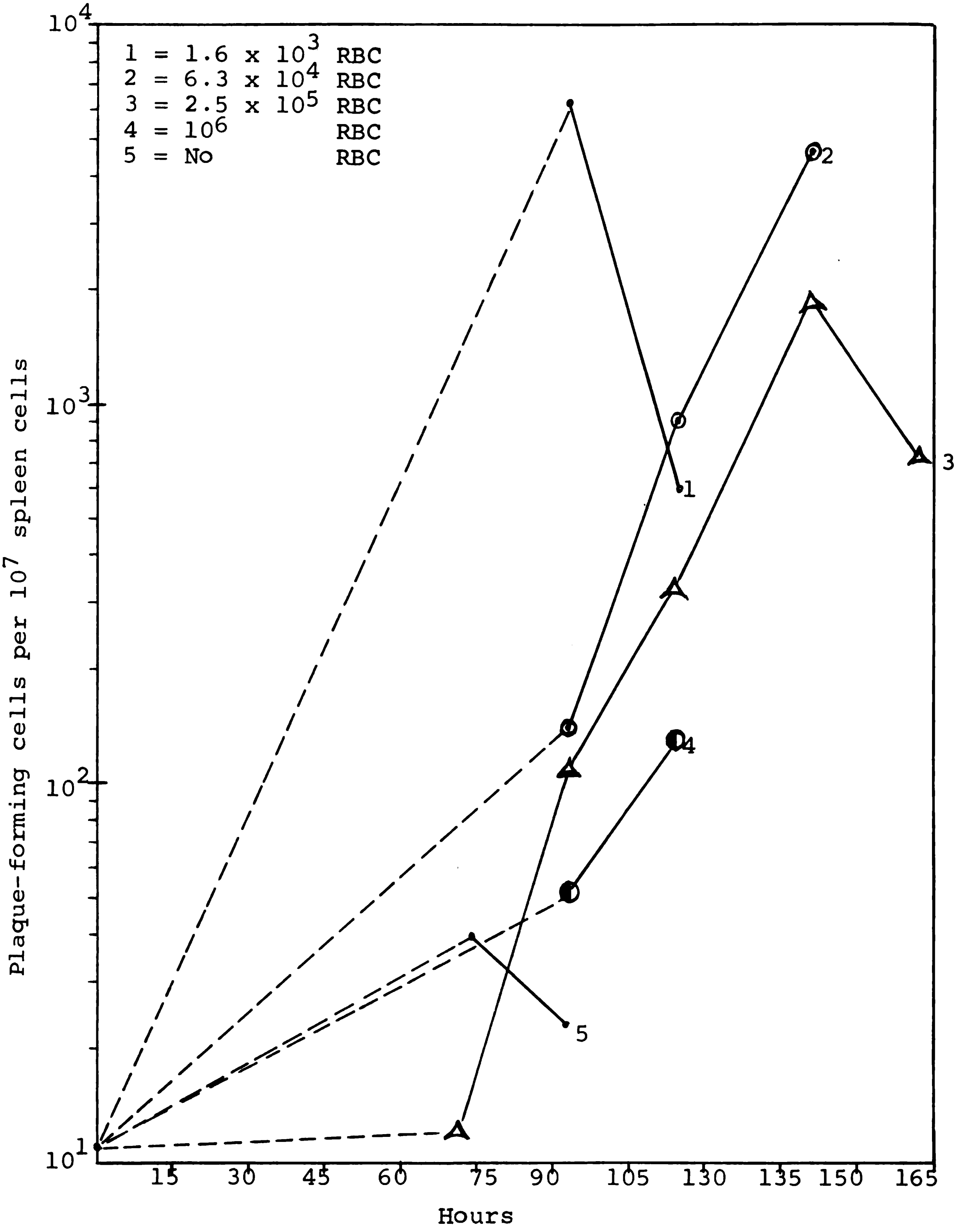

Figure 4. Effect of antigen concentration on the response of spleen cells from rabbit number 32.

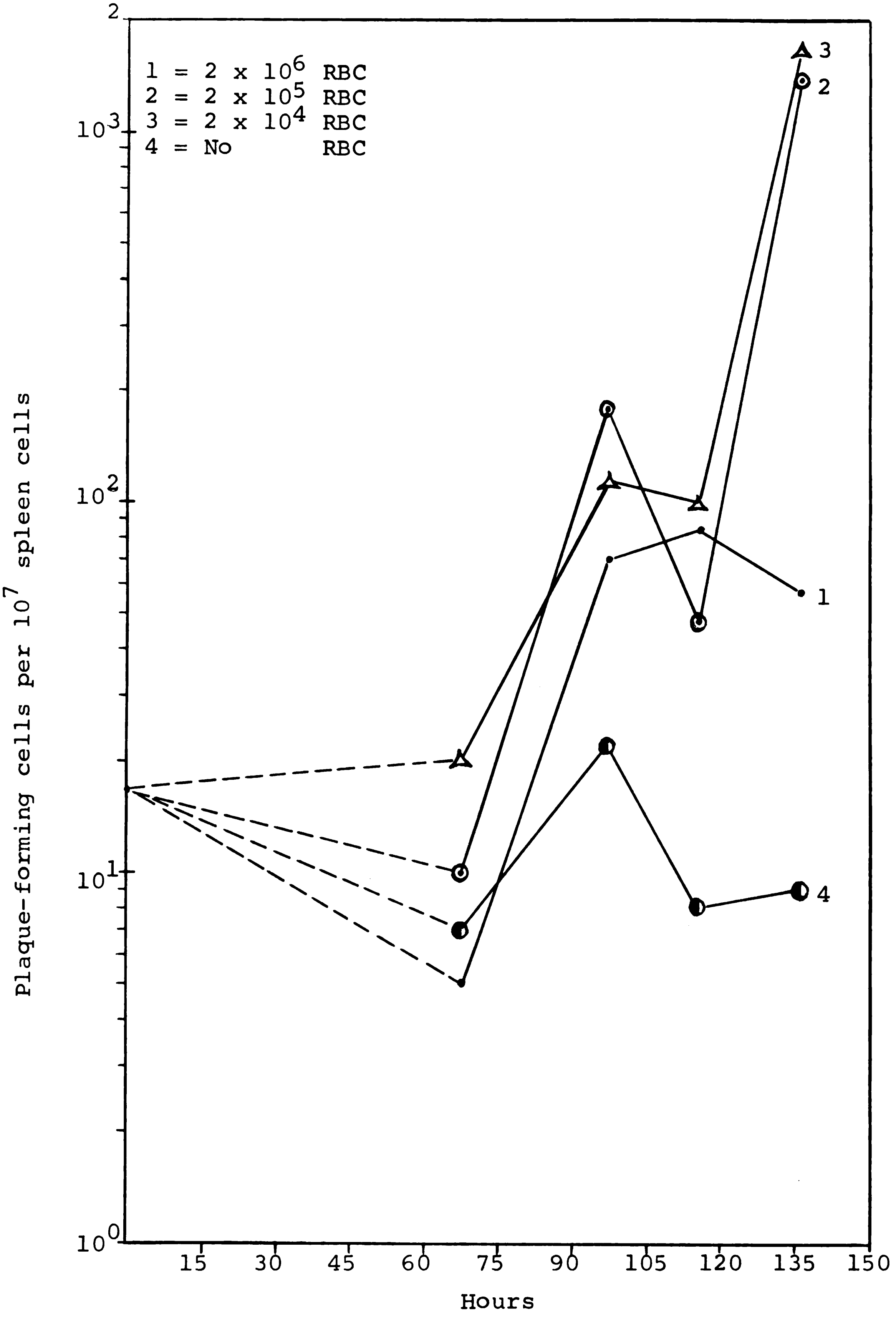

Figure 5. Effect of antigen concentration on the response of spleen cells from rabbit number 41.

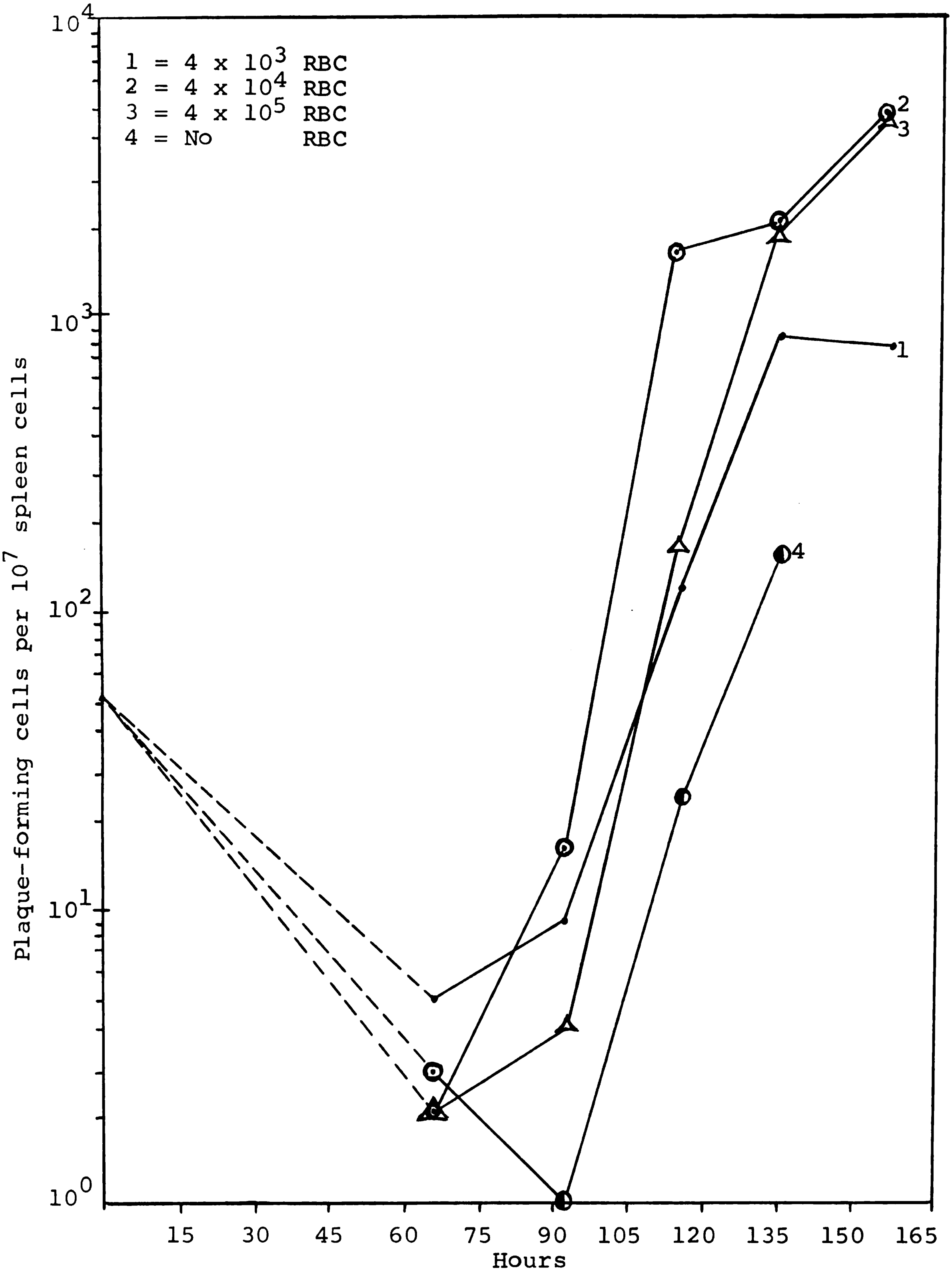

Figure 6. Effect of antigen concentration on the response of spleen cells from rabbit number 42.

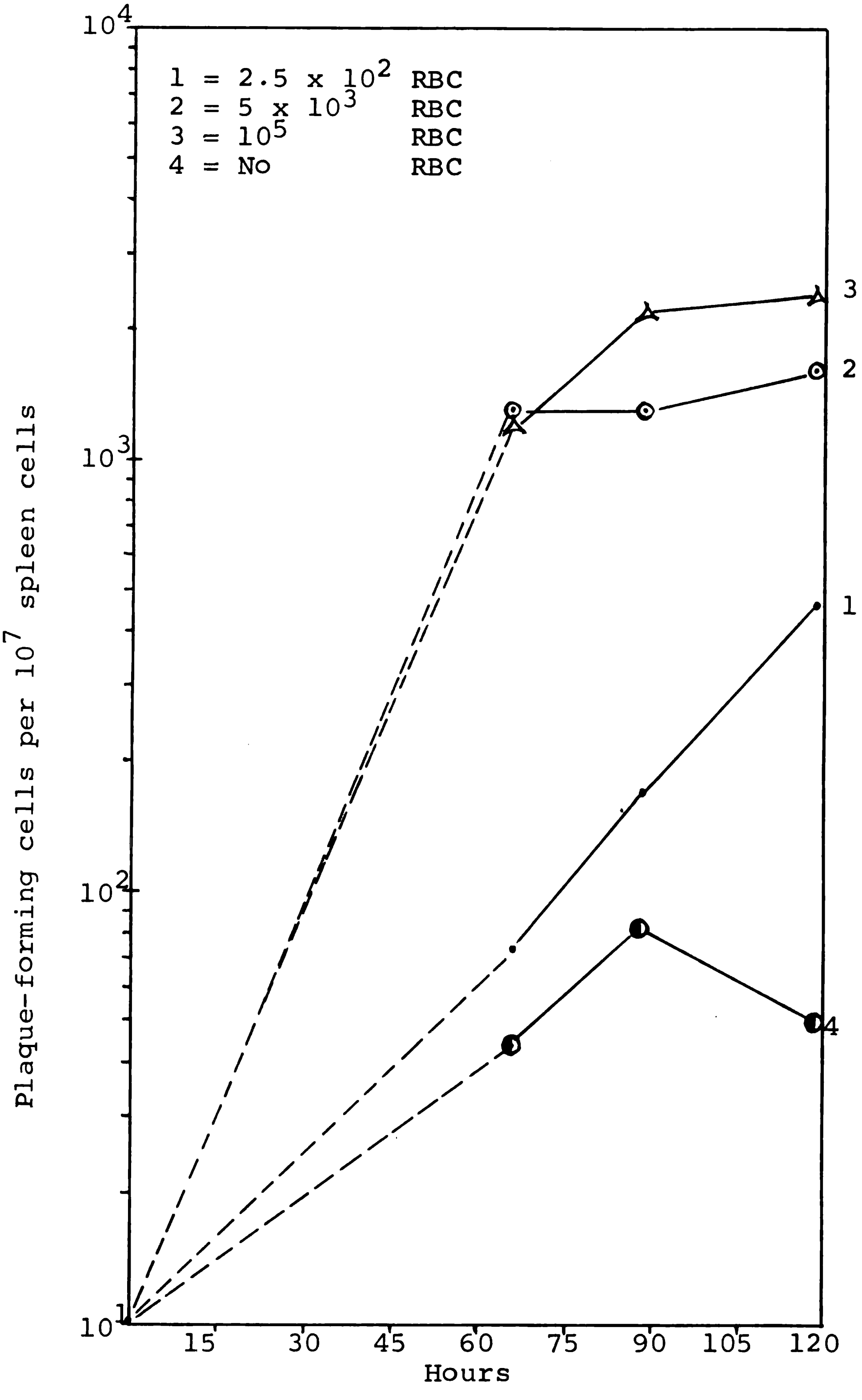

Figure 7. Effect of antigen concentration on the response of spleen cells from rabbit number 43.

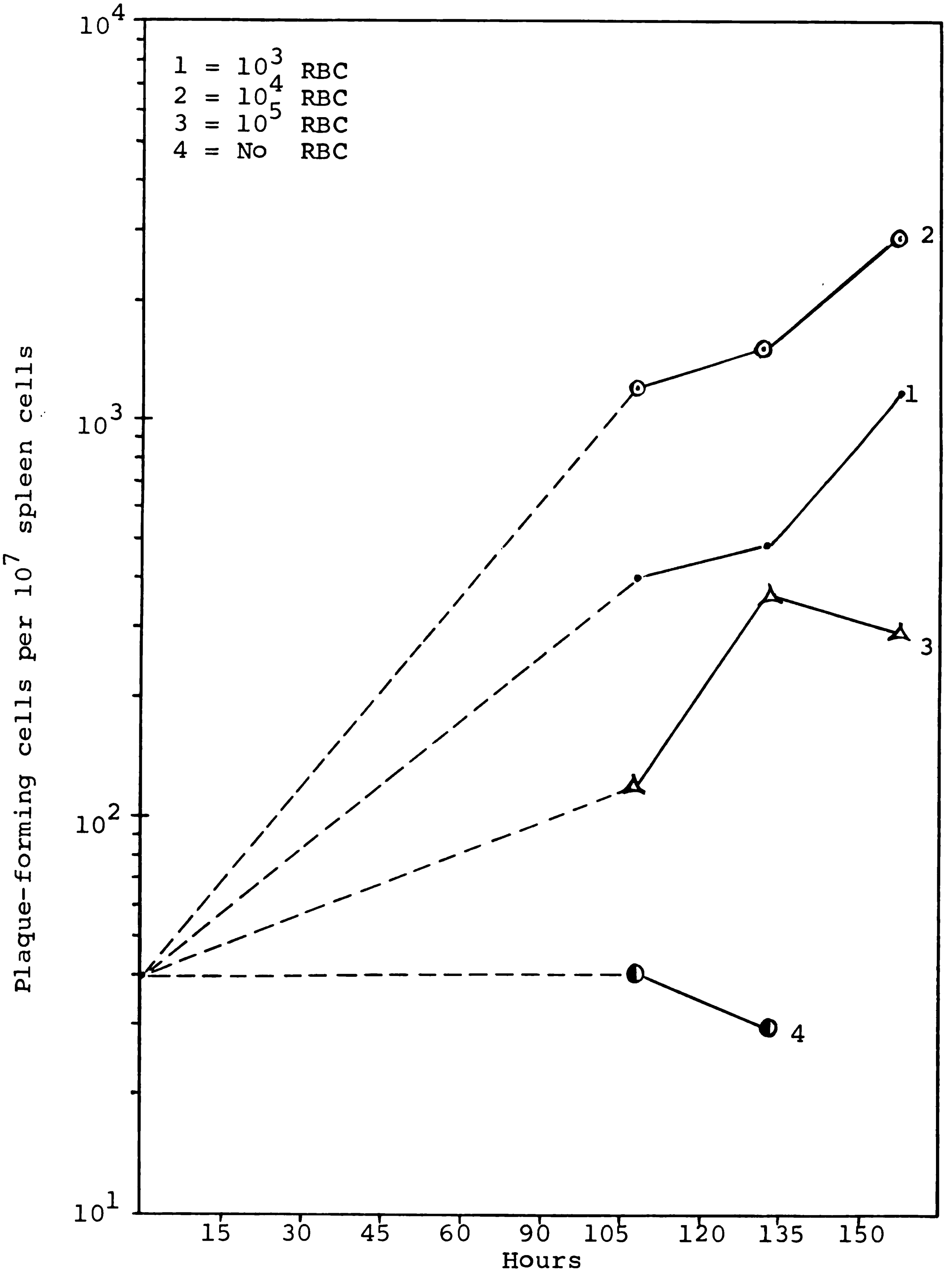

Figure 8. Effect of antigen concentration on the response of spleen cells from rabbit number 45.

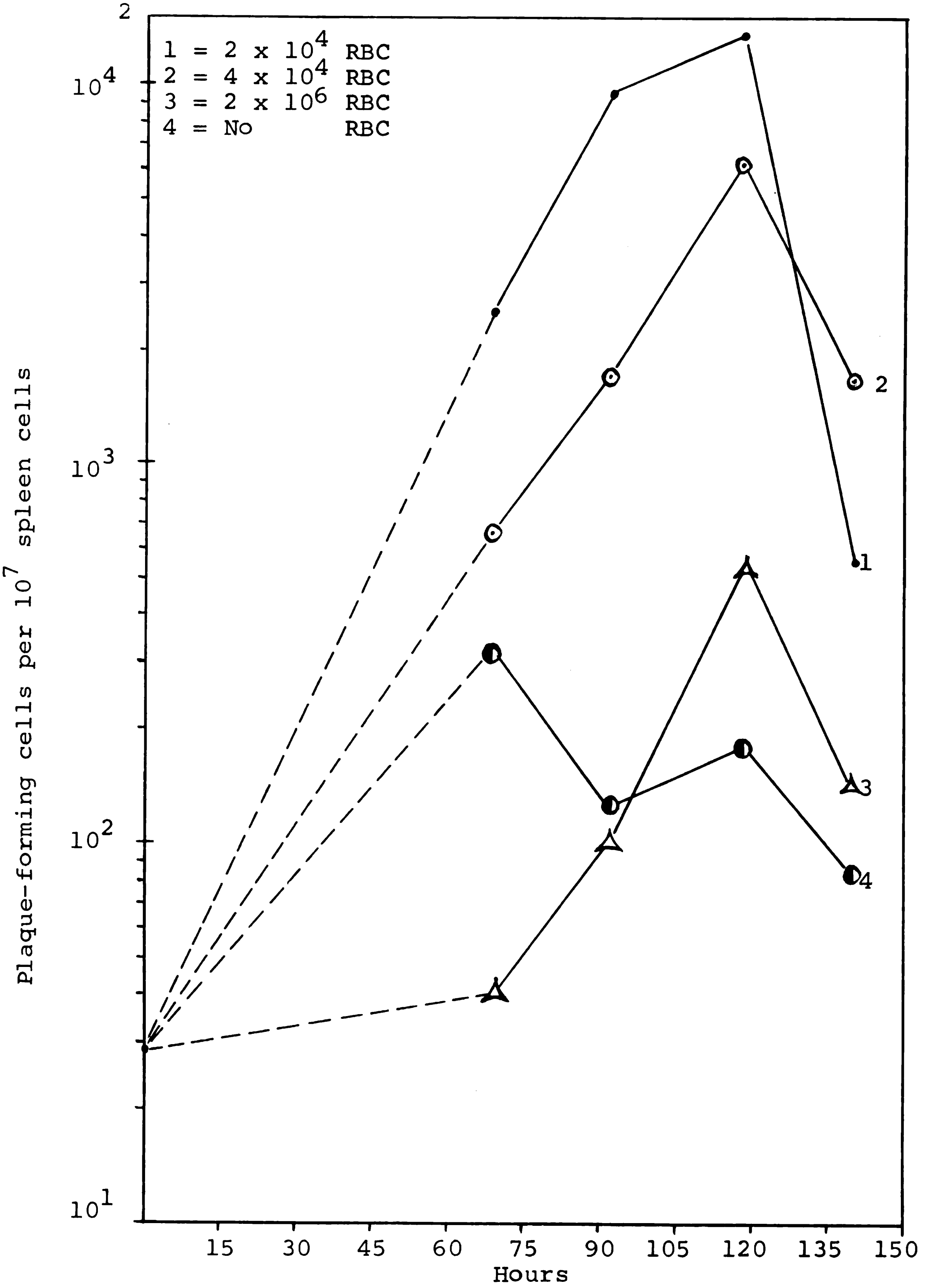

Figure 9. Effect of antigen concentration on the response of spleen cells from rabbit number 50.

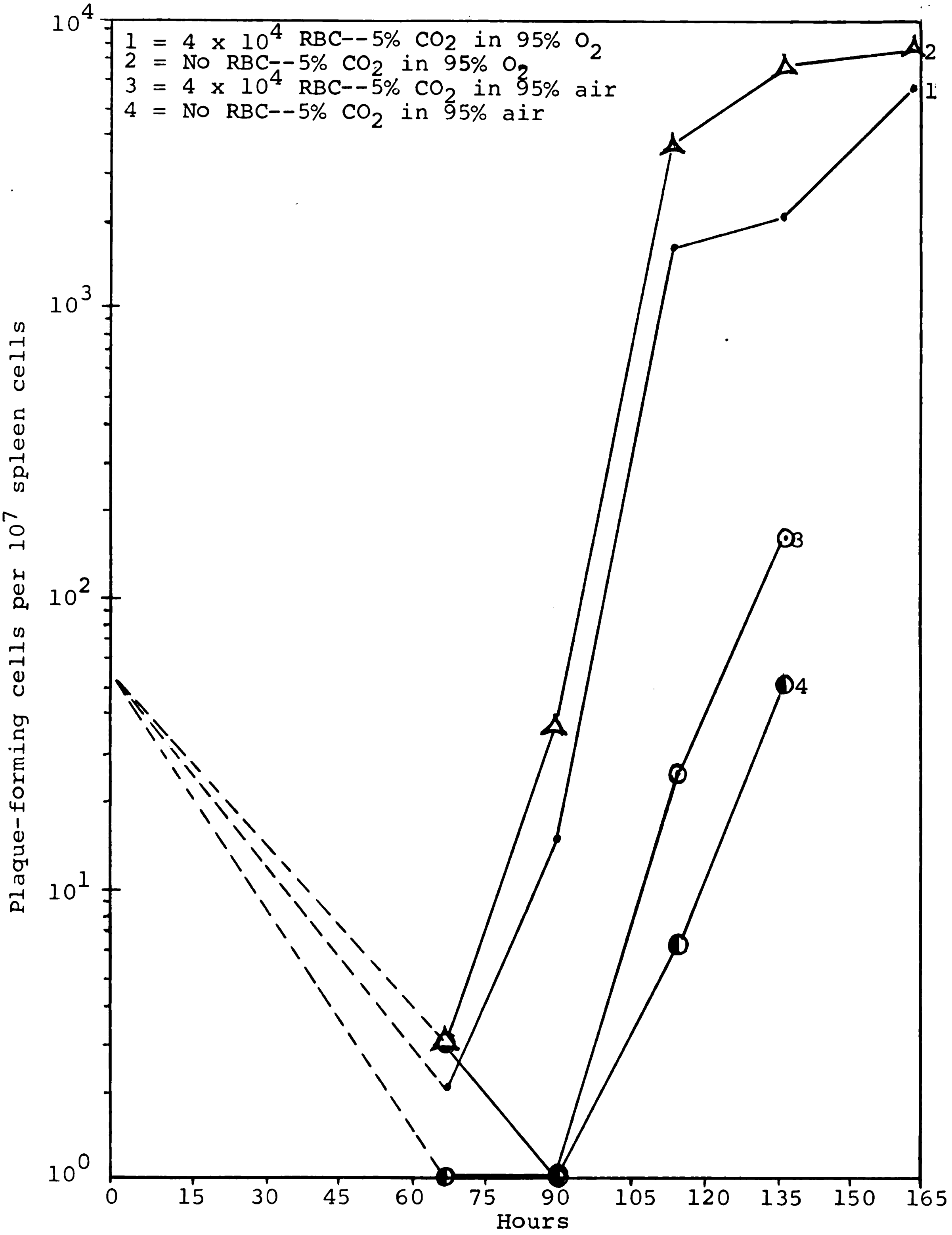

Figure 10. Effect of 5% CO_2 in 95% O_2 and 5% CO_2 in 95% air on the response of spleen cells from rabbit number 42.

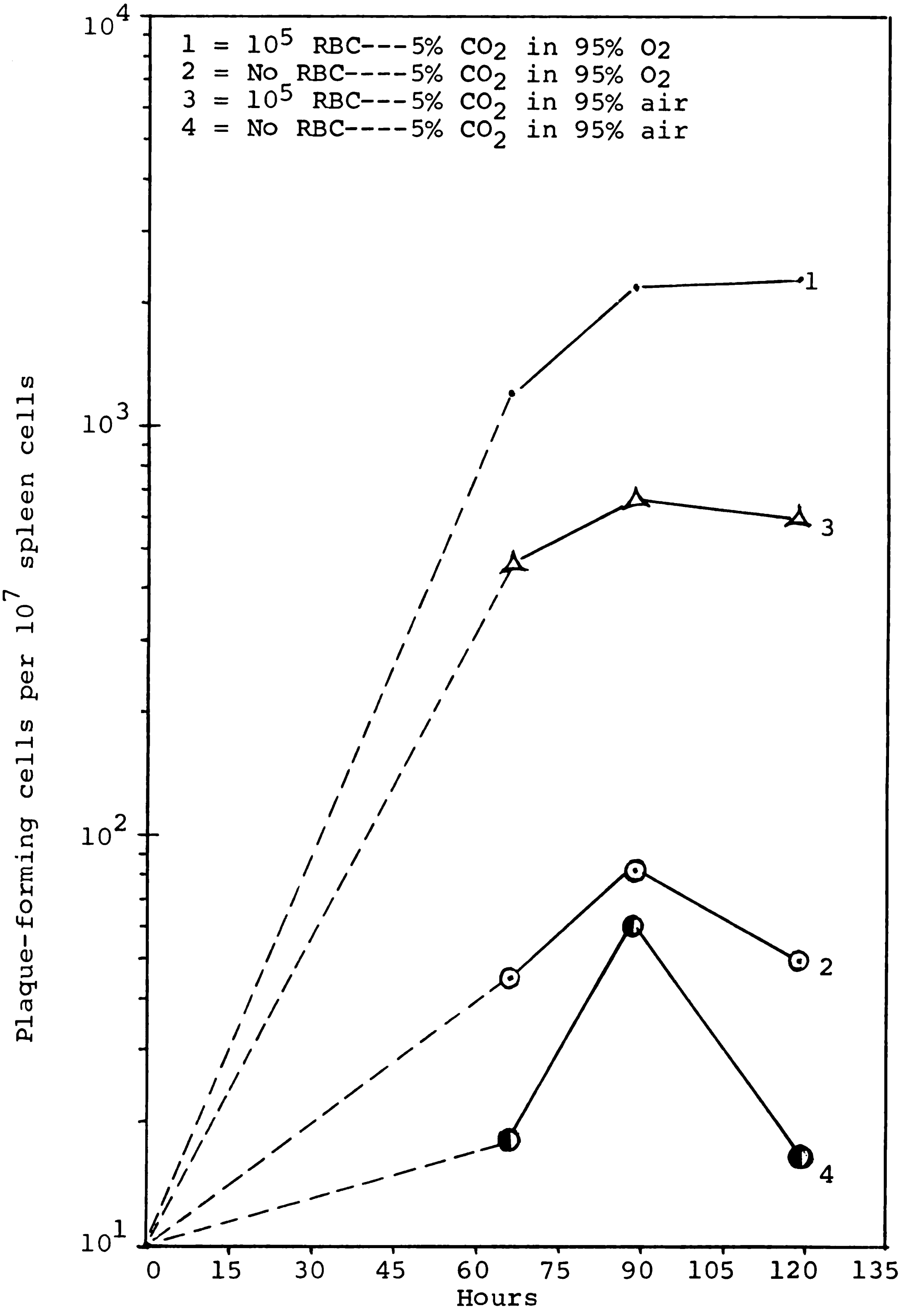

Figure 11. Effect of 5% CO_2 in 95% O_2 and 5% CO_2 in 95% air on the response of spleen cells from rabbit number 43.

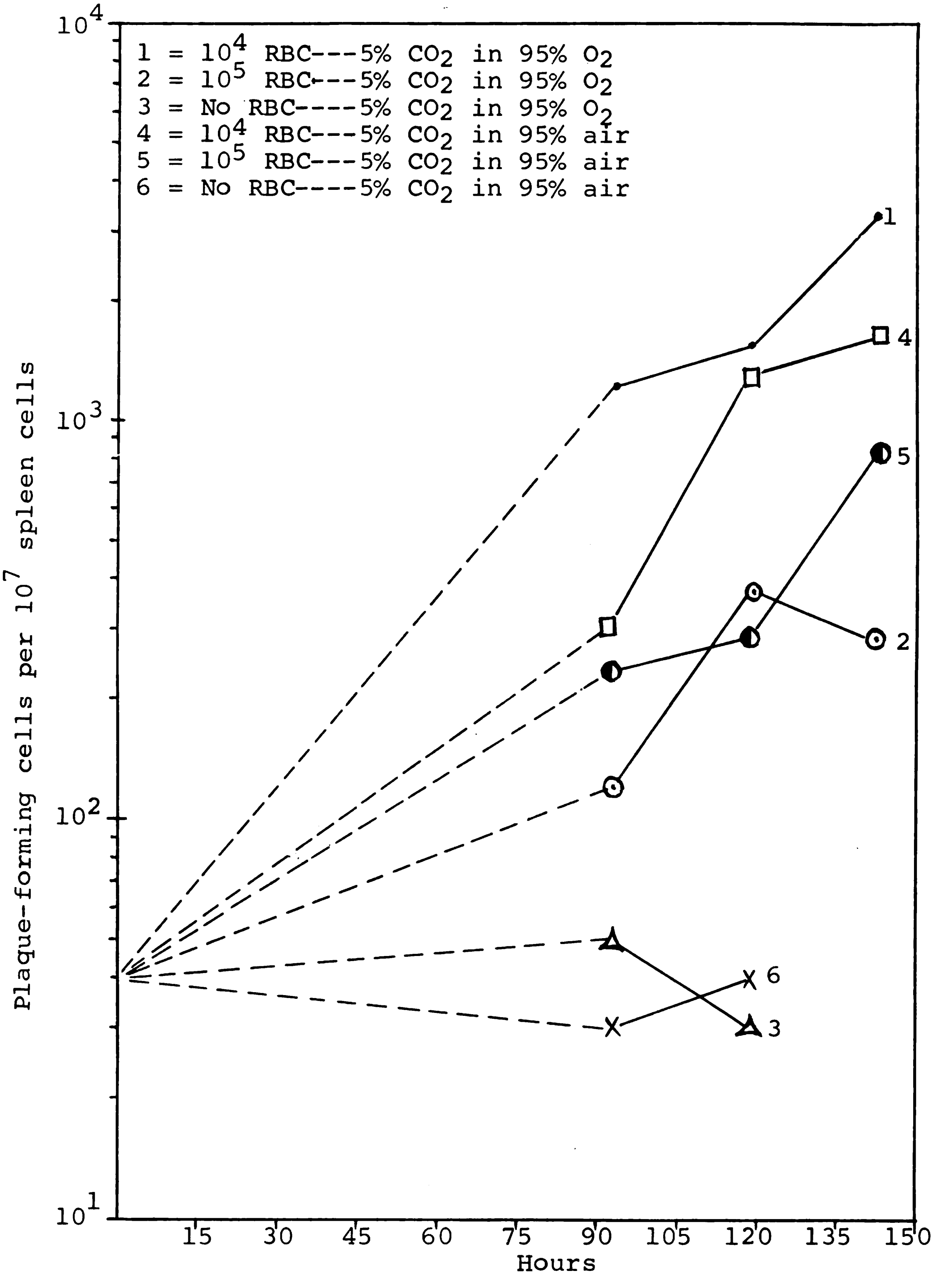

Figure 12. Effect of 5% CO_2 in 95% O_2 and 5% CO_2 in 95% air on the response of spleen cells from rabbit number 45.

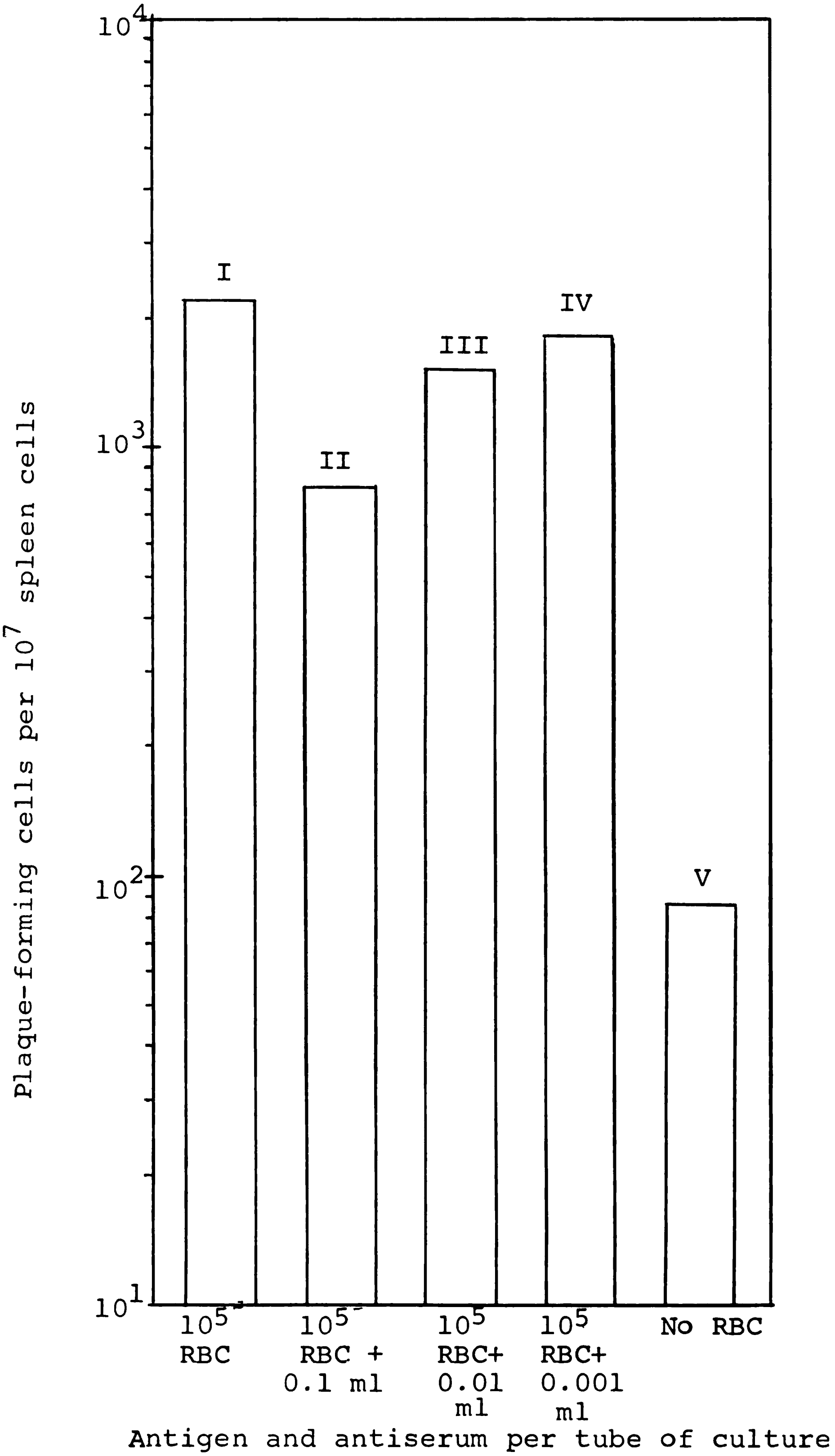

Figure 13. Effect of specific antiserum on the numbers of plaque-forming cells produced by spleen cell cultures from rabbit number 43.

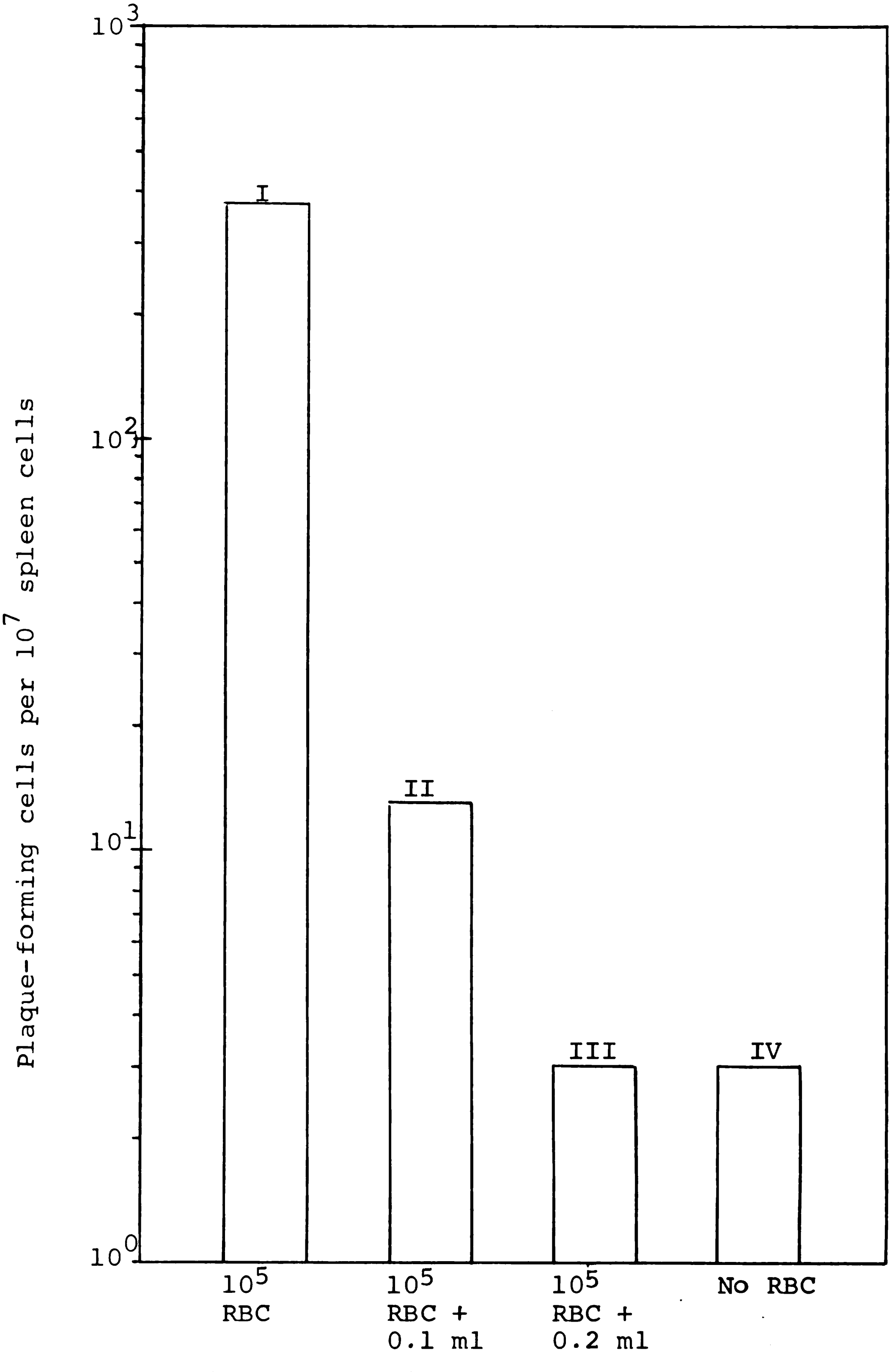

Figure 14. Effect of specific antiserum, added at 48 hours, on the number of plaque-forming cells produced by spleen cell cultures from rabbit number 45.

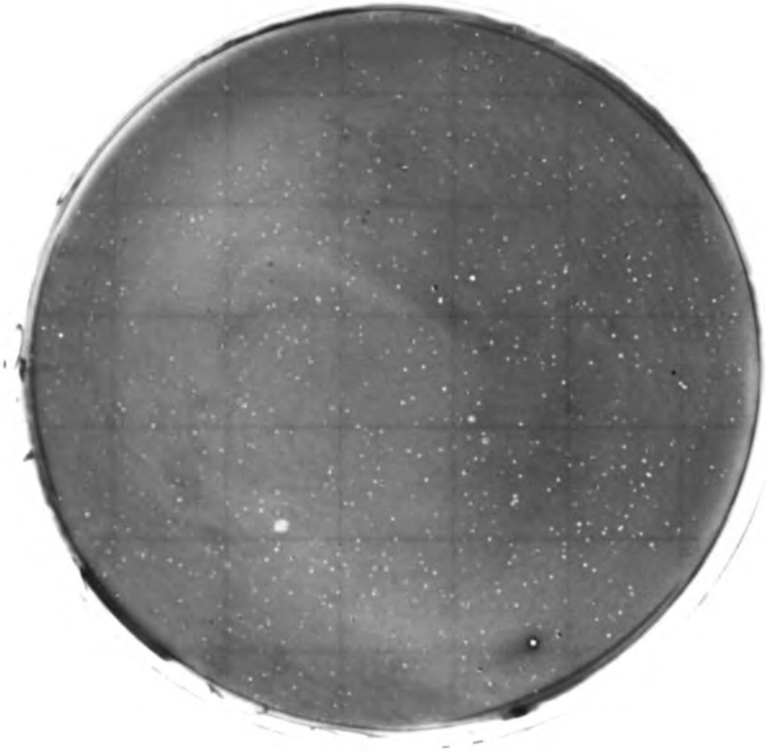

Figure 15. A plate showing plaques produced by antibody-forming spleen cells cultured for 93 hours.

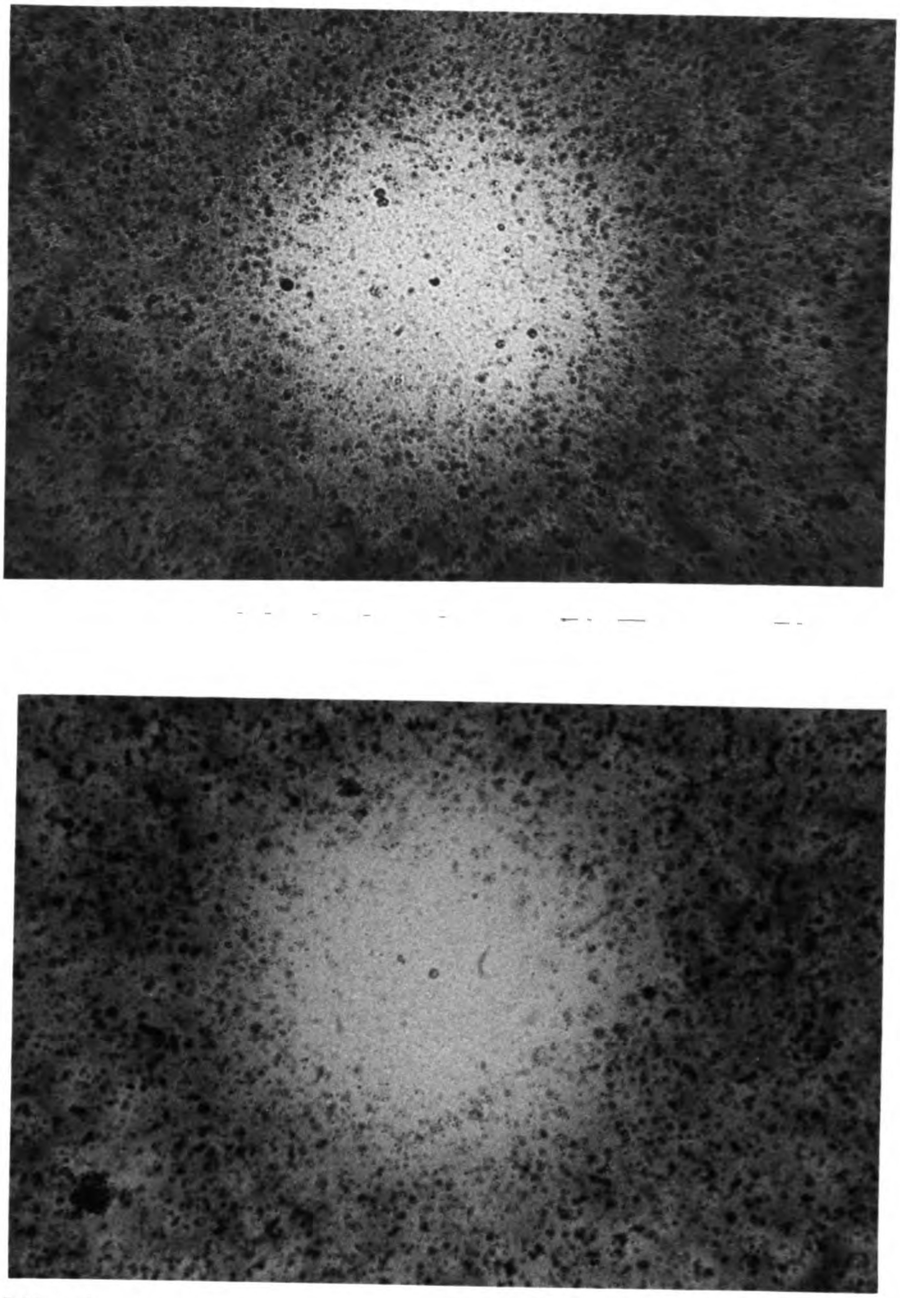

Figure 16. Individual plaques with an antibody-forming spleen cell in the center.

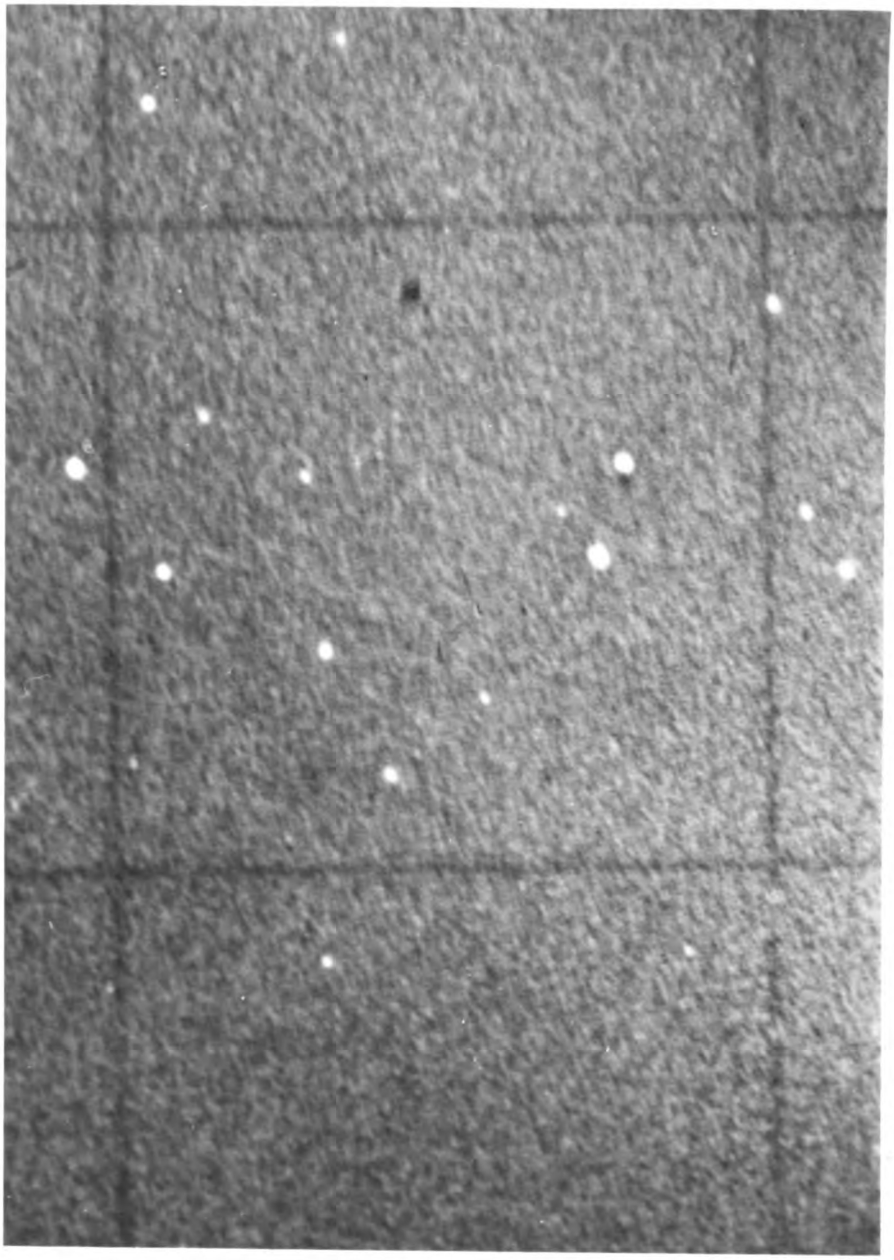

Figure 17. A field demonstrating the various sizes of plaques produced by antibody-forming spleen cells.

DISCUSSION

The secondary response to antigenic stimulation in vivo has recently been described by Coons (11) using current experimental findings to detail his explanation. In general, after the injection of antigen previously stimulated cells, "memory cells," respond by undergoing rapid multiplication and differentiation. After about 48 hours, small amounts of specific antibody can be detected in the cytoplasm of the rapidly multiplying cells. During the next 2 to 3 days the cells show an exponential increase in number and serum antibody concentration. Maximum antibody production is usually achieved between the 3rd and 5th day after which there is a rapid decline.

The response curves found in this study of factors affecting the secondary response in vitro, with respect to the appearance of antibody-forming cells, correlate well with the above description indicating that a true secondary response, analogous to that seen in vivo, was initiated and maintained in vitro.

The induction period of the secondary response in vitro, i.e., the time during which the number of plaque-forming-cells (PFC) did not exceed that obtained at 0 hour, was found to be between 70 and 90 hours. This latent period is in agreement with the results obtained by Nossal and Makela (45); and Uhr *et al*. (69) using in vivo stimulation; Albright and Makinodan (1) using stimulation in vitro followed by intravenous injection of the cells into isologous, irradiated mice; and by Richardson and Dutton (49) using the system employed in this study. The latent period is somewhat longer than that obtained by Hege and Cole (32). These workers, using the Jerne technique for investigation of primary and secondary antibody responses of mice to sheep erythrocytes, detected significant numbers of PFC 1 day following secondary stimulation in vivo. However, the maximum time between primary immunization and secondary stimulation of the mice was only 9 weeks whereas at least 6 months elapsed before the rabbits in this investigation were used. The fact that stimulation was initiated and maintained in vitro may have considerable effect on the time required for the cells to respond. Dutton and Parkhouse (16) have demonstrated that spleen cell cultures from immunized rabbits show a marked reduction in the rate of DNA synthesis during the first 20 hours of culture. If antigen was added to the cell cultures at 0 hour, a similar decline was observed which was followed by a marked rise, apparent by 16 to 24 hours. These workers concluded that the reduction in DNA synthesis was due to the cells adjusting to the environment of the culture in vitro. The 70-to-90-hour induction period by the spleen cells stimulated in vitro may be due, in part, to adjustment of the cells to the new conditions. If 16 to 24 hours are needed for cellular adjustment, only 46 to 76 hours is actually needed for the production of antibody-forming cells. This time would be slightly less than is usually required for stimulation in vivo. That immunocompetent cells could respond to antigenic stimulation in vitro faster than cells in vivo is conceivable in that the cells have been removed from their complex environment in vivo and placed in the presence of an antigen to which they have been immunized to respond. Albright and Makinodan (1) found that the length of the induction period was dependent on the amount of antigen used in vitro. After incubating spleen cells from immunized mice with antigen in vitro, these workers injected the mixture into irradiated recipient mice. With very low numbers of red blood cells they obtained an induction period nearly twice as long as was seen when higher numbers of red blood cells were used. No such effect was found in this investigation with cultured spleen cells. Antigen at very low concentration produced a reduced number of PFC but the induction period was unchanged. This difference in time between the two induction periods may be explained by the fact that following brief incubation of antigen with spleen cells in vitro, Albright and Makinodan (1) diluted the antigen-spleen cell combination by injecting it into the recipient mice. Any unstimulated cells would have little chance to come in contact with the antigen again. Thus, the few cells that were initially stimulated would require more time to produce a detectable level of antibody. In the stimulation in vitro studied here, the particulate red blood cell antigen was added at 0 hour and remained in contact with the spleen cells for the duration of the culture. The persistence of antigen may allow more spleen cells to become stimulated. Therefore, only the maximum number of PFC might be affected rather than the time required for their detection.

That the amount of antigen used for stimulation has a direct effect on the antibody response has been known for some time and has been investigated by numerous workers (1, 47, 63, 64, 65, 69). O'Brien <u>et al</u>. (47) found that the minimum antigen concentration of bovine serum albumin or diphtheria toxoid which consistently stimulated a detectable antibody response from rabbit lymph node fragments was 0.01 μg per milliliter of medium. Uhr <u>et al</u>. (69) found that at least 6×10^2 bacteriophage Φx174 were necessary to initiate a detectable secondary response in guinea pigs. Uhr also found that in their particular system, the rate of antibody formation was independent of antigen over a 10^4 range. Taliaferro (65) compared the antigen required for detectable primary and secondary responses in rabbits. They found that 10^2 sheep erythrocytes stimulated a peak secondary hemolysin titer of 100 whereas 10^5 erythrocytes were necessary to obtain the same titer in non-immunized animals. Antigen concentration of $10^{10.6}$ red blood cells elicited nearly the same titer in both non-immunized and immunized animals. Albright and Makinodan (1) used antigen concentrations ranging from 2×10^3 to 2×10^8 rat red blood cells per 2×10^7 mouse spleen cells. Their results showed that high antigen concentrations produced a high anamnestic response and a shorter induction period. In addition, they found that the rate of antibody synthesis was identical over a 10^4 range of antigen which is in agreement with Uhr (69).

In this study, the results from stimulating the spleen cells using a wide range of antigen concentration varied somewhat. Very low numbers of red blood cells produced a reduced number of PFC. Albright (1) has suggested that both the amount of antibody synthesized per cell as well as the number of cells actively synthesizing antibody are affected by low antigen doses. That fewer cells are involved in the response has been found in this investigation. No information regarding the amount of antibody produced by each cell was obtained as no difference in the size or clarity of plaques produced by the cells was seen.

Numbers of erythrocytes ranging from 10^3 to 4×10^5 per 10^7 spleen cells gave maximum stimulation in vitro. The response varied so much from one rabbit to another that no one optimum antigen concentration was found. On occasion, spleen cells stimulated with 10^3 erythrocytes achieved maximum plaque production earlier than cultures with higher numbers of red blood cells. This response was not regularly seen and no explanation of its occurrence is known.

When spleen cell cultures were stimulated with 10^6 sheep red blood cells the production of antibody-forming cells was inhibited. Sercarz and Coons (57) obtained inhibition of antibody-producing cells in mice given large doses of bovine serum albumin. Rather than basing the immunological response on the presence of free antibody in the serum, these workers used the fluorescent technique to detect antibody-containing cells. Their results indicated that failure to detect antibody following injection of large amounts of antigen is due to intracellular inhibition of the competent cells rather than to neutralization of released antibody by the excess antigen, i.e., the competent cells never produce specific antibody. Brooke and Karnovsky (6) and Sado _et al_. (53) have reached similar conclusions. The results of this study lend support to these findings in that the Jerne technique detects specific antibody-producing cells, not free antibody in the culture medium.

Maximum numbers of PFC were usually obtained on the 4th and 5th day of culture after which their numbers rapidly declined. These results agree with the recent work of Hege and Cole (32) and with the earlier work of Nossal and Mäkelä (39, 45) and Richardson and Dutton (49). The decline in PFC after peak response may be influenced by exhaustion of the culture medium. Extending the culture time of the spleen cells after peak response was not attempted in the investigation. However, the same abrupt decline in antibody production was observed by Taliaferro and Taliaferro (64) using intact rabbits where essential nutrients are not limited and by Jerne and Nordin (33, 34) using intact mice. Some investigators think that the cellular differentiation leading to the formation of antibody-producing cells is a "suicidal" path (40). Makela and Nossal (39) demonstrated that mature antibody-producing plasma cells did not incorporate ^{3}H-thymidine and concluded that these cells were nondividing and apparently short-lived. Thus, the simple depletion of essential nutrients from the culture medium may not be the whole explanation. What may occur is death or degeneration of the expended cells.

In this study plaques from 0.1 to 0.6 mm in diameter were formed by cultured antibody-synthesizing cells that appeared several days after antigenic stimulation in vitro. Antibody-synthesizing cells that have been stimulated in vivo produce plaques as varied in size. That this difference is not due to synthesis of 19S and 7S antibody by the various cells is suggested by two lines of evidence. First, the majority of the antibody produced by rabbits to sheep erthrocytes has been found by most investigators to have a sedimentation constant of about 19, i.e., 19S antibody (36).

Second, several investigators (32, 44, 52) have found that the unmodified Jerne plaque technique detects only cells synthesizing 19S antibody. Sterzl (61) and Dresser and Wartis (12) have recently demonstrated that by using specific antiglobulin serum 7S antibody-producing cells can be detected by the Jerne technique. Presumably then, the variation in plaque size is due to the amount of antibody being synthesized, the rate at which it is released from the cell, or the degree of diffusion through the agar.

There is little information pertaining to the effect of oxygen or air on the growth of cells in tissue culture and even less on the effect they have on antibody production. Work by Trowell (67, 68) suggested that there was no difference in the efficiency of the two culture atmospheres for growing cells in vitro. Jones and Bonting (35) found that respiratory enzymes, glycolysis, and finally growth was inhibited when embryonic tissue was grown in oxygen. Similar results were obtained by Zwartouw and Westwood (72) using established cell lines. In this investigation, the two atmospheres had no consistent effect on the number of PFC obtained from the spleen cell cultures. Cells cultured in 95% oxygen did produce more distinct zones of hemolysis than did cells cultured in air. This may indicate that oxygen has some enhancing effect on protein synthesis.

In this study of antigenic stimulation in vitro, a preliminary investigation of additives to the culture medium was carried out. Sodium pyruvate, nonessential amino acids, and glutamine were used. All spleen cell cultures incubated in the presence of the three additives produced higher numbers of antibody-forming cells than did cultures without the additives. The increased number of antibody-forming cells from the cultures with the additives can be attributed to a supplement of the biochemical requirements of the spleen cells. In one experiment only pyruvate was added to the culture medium to determine its effect on the numbers of antibody-synthesizing cells. The number of PFC increased significantly as compared to the number from cultures without the additive. Eagle has established that cultures of certain cells require exogenous pyruvate when population densities are low (19). The increase in PFC with pyruvate may result from increased metabolic rate and mitotic division of the spleen cells. On the other hand, it is known that phagocytosis and digestion of antigen by lysosomal enzymes are energy-dependent processes. Pyruvate may enhance antigenic stimulation of the immunocompetent cells rather than increase mitosis.

To determine what effect a supplement of amino acids would have on antigenic stimulation, nonessential amino acids were added to the medium (1) alone, (2) with pyruvate, and (3) with pyruvate and glutamine. In all experiments an increased number of PFC was obtained, as compared to PFC in unsupplemented medium. Although Eagle (19) found only 13 amino acids essential for the growth of cultured cells, he noted enhanced growth in some cell cultures when the medium was supplemented with nonessential amino acids. The increase in PFC noted in this study might be anticipated in that high levels of amino acids are required for rapid cell multiplication. Also it is known from studies with radioactive amino acids that antibody is synthesized de novo from amino acid pools (60). If amino acid concentration was limiting in unsupplemented medium, an increased number of PFC would ensue with the nonessential amino acid supplement.

That glutamine plays a part in the production of higher numbers of antibody-forming cells might be anticipated from its recognized role in metabolism. It serves cultured cells as a specific precursor for nucleic acids, protein, and some nonessential amino acids. That glutamine increased the production of antibody-forming cells was demonstrated in this study by the higher number of PFC obtained from cell cultures with all three additives as compared to those with only pyruvate and nonessential amino acids. The increased plaque production was seen in all cell cultures with the three additives. Such was not the case, however, when glutamine alone was added to the cell cultures.

With glutamine alone, three effects were obtained; (1) inhibition, (2) no effect, and (3) enhancement. First, cell cultures with antigen alone and antigen plus glutamine produced low numbers of PFC whereas cell cultures with no antigen with and without glutamine produced considerably more PFC. This response was obtained at a time when the cell cultures were not responding to antigenic stimulation. Although the difficulty was not identified, it was believed to be due to some foreign substance in the distilled water. This substance seemed to stimulate the cell cultures non-specifically to produce antibody. The low number of PFC from cell cultures with antigen with and without glutamine may be analogous to the results obtained from cell cultures with high numbers of sheep red blood cells, i.e., an overloading of the spleen cells with specific plus non-specific stimulus. The cell cultures with no antigen with and without glutamine could therefore produce more PFC because the sheep erythrocyte antigen was not present.

In the second type glutamine had no effect on the response of the stimulated spleen cells. The maximum number of antibody-producing cells was nearly the same from stimulated cell cultures with and without glutamine and the response occurred at the same time. In addition, low numbers of PFC were obtained from cell cultures with no antigen with and without glutamine.

The third effect obtained from the addition of glutamine was a significant increase in the number of PFC. All cultures with antigen plus glutamine produced nearly 4 times as many PFC as did cultures with only antigen. In these experiments, glutamine exhibited a true stimulatory effect on the ability of the cell cultures to produce antibody-forming cells. An explanation of the difference seen in these latter two responses is not known.

Various concentrations of glutamine were also used. Cell cultures with twice as much glutamine produced more PFC than did replicate cultures with normal glutamine concentration. Cell cultures with 1/5 the concentration produced fewer antibody-forming cells. When glutamine was added to the cell cultures at 48 hours, fewer PFC were produced than from cultures with glutamine at 0 hour. This may indicate that the principle effect of glutamine is to increase DNA synthesis.

The inhibition of antibody-producing cells by specific antiserum that was found in this study is in agreement with the work of Rowley and Fitch (51) and Möller and Wigzell (44). However, certain differences are apparent. The results of this investigation show a marked inhibition by specific antiserum of the secondary antibody response in vitro. The degree of inhibition was directly related to the concentration of antiserum added to the cell cultures and was more pronounced when the antiserum was added at 48 hours. Rowley and Fitch (51) were able to suppress the primary antibody response of rats to sheep erythrocytes by passive immunization with specific antiserum but were unable to suppress the secondary response by the same treatment. Identical results were obtained when spleen cells were removed from normal and immunized rats, incubated with specific antiserum, and transferred to x-irradiated hosts. Following injection of antigen into these host animals, the normal spleen cells failed to produce antibody while the spleen cells removed from immunized animals produced antibody at detectable levels. Perhaps their inability to suppress the secondary response was due to the use of in vivo techniques. As mentioned above, the degree of inhibition of the response in vitro was dependent on the concentration of antiserum added. The antiserum would be more concentrated in vitro and would not be subjected to dilution by injection into living animals. In addition, the fact that a higher degree of inhibition of PFC was obtained when antiserum was added at 48 hours may indicate that the antibody-producing cells are more susceptible to specific antiserum after they have been stimulated by antigen. Injection of specific antiserum 1 or 2 days after secondary stimulation was not reported by Rowley and Fitch (51). Based on their results these workers concluded that there are two types of cells involved in the immune response. "Potential antibody-forming cells" cannot respond to antigen in the presence of specific antibody while "antibody-forming cells" from immunized animals can respond to antigen in the presence of specific antibody. The primary response was not studied in this investigation, thus no such distinction of cells was obtained.

Möller and Wigzell (44), using specific 19S and 7S antibody, confirmed and extended the experiments of Rowley and Fitch. Utilizing the Jerne technique these workers found that the inhibiting efficiency of 7S antibody on the primary response was about 100 to 200 times greater than that of 19S antibody. The suppression of the number of PFC was also dependent on the amount of antiserum administered. This may indicate that the concentration of antibody is a critical factor.

Brent and Meduwar (5) have suggested that the inhibition of antibody formation by specific antibody occurs directly at the cellular level rather than by combining with the antigenic determinants. The results obtained from spleen cell cultures with antiserum added at 48 hours lend support to this idea because it is assumed that by 48 hours of culture, antigen has already been taken up by the immunocompetent cells. The mechanism of this type of antibody inhibition remains to be elucidated.

The specificity of the antibody synthesized in vitro was demonstrated by testing it against red blood cells from 5 species. All of the 7 day culture mediums tested showed specific lysis of sheep red blood cells. A weak reaction was obtained with chicken red blood cells but horse, bovine, and human red blood cells failed to react. Antigenic determinants common to both the sheep and chicken red blood cells presumably would account for the reaction.

In conclusion, it has been demonstrated that the secondary antibody response initiated and maintained in vitro corresponds in many respects to the secondary antibody response in vivo. Many questions remain to be answered but with the use of cultured cells the immunological response can be studied in detail. As the technique is refined and culture conditions improved, a more precise understanding of antigenic stimulation, antibody production, and factors affecting the two may be gained.

SUMMARY

Experimental conditions affecting secondary antigenic stimulation in vitro of spleen cells from immunized rabbits have been investigated in this study. The Jerne plaque technique was used for detection of the antibody-forming spleen cells.

It was found that no increase in the number of antibody-forming cells occurred during the first 2 or 3 days of culture. Between the 4th and 6th day the response appeared to show an exponential increase to a maximum number of plaque-forming cells followed by a rapid decline. The maximum response of spleen cells from 12 rabbits ranged from 160 to 21,050 plaque-forming cells per 10^7 spleen cells. The induction period, time of maximum response, and decline of plaque-forming cells in vitro found in this study correlate well with the secondary antibody response in vivo.

The number of sheep red blood cells (antigen) added to the spleen cell cultures proved to be of primary importance for obtaining maximum stimulation. Maximum plaque formation occurred when 10^3 to 4 x 10^5 sheep red blood cells per 10^7 spleen cells were added to the cell cultures. Stimulation of the spleen cells with less than 10^3 red blood cells resulted in the production of low numbers of antibody-forming cells. Addition of 10^6 or more red blood cells inhibited the production of antibody-forming cells.

No consistent difference in the number of plaque-forming cells was evident when the cell cultures were incubated in atmospheres of 5% CO_2 in 95% O_2 and 5% CO_2 in 95% air. More distinct hemolytic plaques were produced by antibody-forming cells cultured in 5% CO_2 in 95% O_2.

Spleen cells cultured in the presence of sodium pyruvate, nonessential amino acids, and glutamine consistently produced a higher number of antibody-forming cells than did cultures without the three additives. When only pyruvate was added to the cell cultures, the number of plaque-forming cells increased significantly as compared to the number from cultures without pyruvate. When nonessential amino acids were added to the medium with and without pyruvate, a similar increase in the number of plaque-forming cells occurred. With glutamine alone, three effects on the number of plaque-forming cells were obtained: (1) inhibition, (2) no effect, and (3) enhancement.

A marked inhibition of the number of antibody-forming cells was obtained when specific antiserum was added to the culture medium. The degree of inhibition was directly related to the concentration of the antiserum added. The effect was more pronounced when the antiserum was added 48 hours after the culture was prepared.

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