THE RATE OF DIVISION OF ANTIBODY-FORMING CELLS DURING THE EARLY PRIMARY IMMUNE RESPONSE*

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There is conflicting evidence about the relative roles of division and "recruitment" of responding cells during the early primary immune response. The cells primarily releasing "19S" hemolysin for sheep erythrocytes may be enumerated using the plaque-forming cell technique developed by Jerne and Nordin (1, 2) and independently by Ingraham (3). An exponential increase in plaque-forming cells occurs during the early primary response; doubling times vary from about 5 to 10 hr depending on the dose of antigen and other experimental variables (4–14).

A method using mitotic blocking agents to estimate cell cycle times of antibody-forming cells, in vivo and in vitro, is presented. Results with this method indicate that the increase in numbers of antibody-forming cells which occurs during the early primary response is due primarily to exponential division of responding cells. Increased responses produced by larger antigen doses or adjuvant appear to result from an increased rate of division of responding cells. These findings are contrary to the view that the observed increase in antibody-producing cells can be attributed to continued activation or recruitment of potential antibody-producing cells (9–14).

Materials and Methods

Young adult Sprague Dawley rats were used. The antigen was washed whole erythrocytes prepared from sterile sheep's blood. A single sample of blood stored in Alsever's solution was used in each experiment both for immunization and for titrating immune responses. The sheep erythrocytes were washed four times in large volumes of freshly prepared pyrogen-free saline and used immediately. Doses of sheep erythrocytes were standardized by cell

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count. *Bordetella pertussis* vaccine was used to enhance the response to sheep erythrocytes and will be referred to as adjuvant. The vaccine (Lilly, strain 37790, lot T-59187), containing about 50 opacity units per ml and Merthiolate 1:10,000, was kindly supplied through Dr. H. Campbell, Jr., Biological Development, Eli Lilly and Co., Indianapolis, Ind. The adjuvant was mixed with antigen or injected separately as indicated. All injections of antigen and adjuvant were into the lateral tail vein.

Plaque-Forming Cells.—The method of Jerne and Nordin (1) for demonstrating release of antibody by single cells in agar plates was used with minor modifications (15). Each spleen was minced and passed through a wire gauze screen using a pestle and 4.0 ml of tissue culture medium. Repeated pipetting of the cell suspension caused additional dispersion of cells. The volume of the suspension was brought to 5.0 ml and tissue fragments were removed by low speed centrifugation for 30 sec. 0.1 ml of an appropriate dilution of the spleen cell suspension and 0.1 ml of a suspension containing 2×10^8 sheep erythrocytes mixed with 2.0 ml of agar–tissue culture medium were layered over a thin bottom layer of solidified agar–tissue culture medium in plastic Petri plates 9.0 cm in diameter. The plates were incubated 1.5 hr at 37°C; 2.0 ml of normal rat serum diluted 1:3 with saline was added as complement; the plates were incubated 1.5 hr at 37°C, washed, and counted immediately. Except for very early responses when spleens contained few plaque-forming cells, the total number of spleen plaque-forming cells for each animal was calculated from counts on duplicate plates using dilutions of spleen cell suspensions which gave about 100–250 plaques per plate.

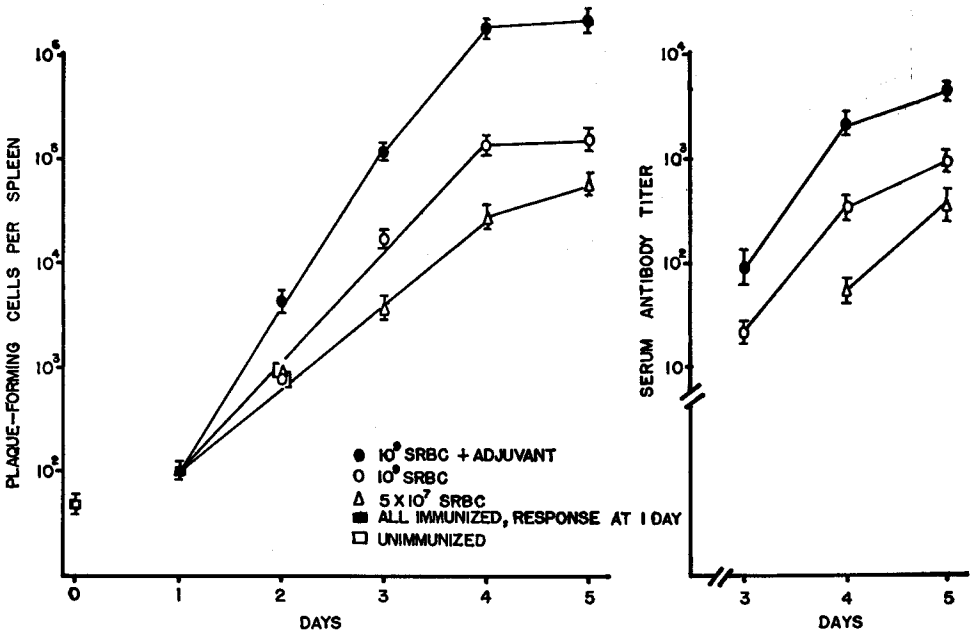
The calculated total number of plaque-forming cells per spleen was transformed by taking the logarithm of the number to the base 10. Where means are recorded untransformed, they represent the antilog of the mean of the logarithmically transformed data. All statistical calculations were performed on the logarithmically transformed data. The reasons for preferring the logarithmic to the simple arithmetic scale arise from the fact that the scatter around the untransformed means was grossly dependent upon the mean itself, the higher mean values being attended by greater variability. Logarithmic transformation of the data fully corrected this feature, so that the variance at different response levels was independent of the mean and showed no significant trend.

Antibody Titers.—Titrations for hemolytic and agglutinating antibody to sheep erythrocytes used the double dilution technique with an initial serum dilution of 1:10 (15, 16). During the first 5 days of the primary response to sheep erythrocytes, the agglutinin and hemolysin titers were equal ± one serum dilution using selected end points; the relationship between spleen plaque-forming cells and antibody titers was the same whether agglutinin or hemolysin titers were used. Only hemagglutinin titers are reported. For convenience in comparing the plaque-forming cell and antibody responses, titers were also transformed by taking the logarithm of the number to the base 10. Where mean titers are recorded untransformed, they represent the antilog of the mean of the logarithmically transformed data. All statistical calculations were performed on the logarithmically transformed data. Sera were also titrated for antibody not inactivated by 2-mercaptoethanol; serum diluted 1:5 in saline was added to an equal volume of 0.1 M 2-mercaptoethanol, incubated at 37°C for 30 min, and then titrated for hemagglutinins.

Mitotic Blocking Agents.—Colchicine, 0.5 mg/ml, injectable, was purchased from Eli Lilly & Co. Velban (vinblastine sulfate) lot No. 9EP95, was kindly supplied by Dr. I. S. Johnson, Biology Research Division, Eli Lilly & Co.; fresh solutions of the powder dissolved in pyrogen-free normal saline were prepared for each experiment.

In Vitro Cultures.—Dispersed spleen cells of DBA/2 Jax mice were immunized in vitro with sheep erythrocytes by the methods of Mishell and Dutton (17, 18) or a modification of the method used in this laboratory (19). Responses were measured after 1 to 4 days of culture. Cultured cells, including cells free in the culture medium and cells adherent to plastic, were harvested by scraping dishes with a plastic policeman. A modification of the Jerne technique

using microscopic slides was used for enumerating plaques (18). When responses were measured 4 days after immunization, appropriate dilutions of cultured cells were made so that about 100–200 plaques were present on each slide. Each plaque count recorded for the in vitro cultures is the mean (to the nearest whole number) of counts on 2–4 slides prepared from a pool obtained from duplicate culture dishes. Counts are expressed as the number of plaques or plaque-forming cells per 10^6 cells initially cultured (19). 4 day cultures of normal mouse



TEXT-FIG. 1. Spleen plaque-forming cell and serum antibody responses of rats immunized intravenously with 5×10^7, 10^9 sheep erythrocytes, or 10^9 sheep erythrocytes and adjuvant. The adjuvant was 0.15 ml of *B. pertussis* vaccine given intravenously at the time of immunization. Each point represents the mean of the $\log_{10}$ transformed data ± the standard error of the mean plotted as a vertical bar. The point at day 0 is the mean for 35 unimmunized rats; the point at day 1 is the mean for 32 rats; 8 immunized with 5×10^7 sheep erythrocytes, 16 immunized with 10^9 sheep erythrocytes, and 8 immunized with 10^9 sheep erythrocytes and adjuvant. Each other point is the mean for 14 or more rats.

spleen cells that were not immunized in vitro with sheep erythrocytes had one or fewer "background" plaque-forming cells per 10^6 spleen cells initially cultured.

PRELIMINARY OBSERVATIONS

Responses to Antigen.—The spleen plaque-forming cell and serum antibody responses were measured 1–5 days after immunization for doses of antigen ranging from 10^5 to 10^{10} sheep erythrocytes per rat. Spleens of normal rats contained about 80 "background" plaque-forming cells for sheep erythrocytes; sera contained no detectable antibody to the antigen. Rats immunized with

10^6–10^7 sheep erythrocytes invariably had slightly increased numbers of spleen plaque-forming cells by 3–5 days; there was considerable individual variation in response, and sera contained no or very low titers of antibody. A dose of 5×10^7 sheep erythrocytes approximated the lowest dose of antigen that regularly produced reasonably consistent plaque-forming cell and antibody responses. Maximum responses were produced by 5×10^8–10^9 sheep erythrocytes; increasing the dose to 10^{10} sheep erythrocytes did not appreciably affect the response during the first 5 days. A dose of 10^9 sheep erythrocytes was selected as a "saturation" dose of antigen, i.e., a dose capable of producing a maximum response.

TABLE I

Adjuvant Enhancement of Responses to 10^9 Sheep Erythrocytes

	Plaque-forming cells per spleen* mean of $\log_{10} \pm$SE			Serum antibody titers* mean of $\log_{10} \pm$ SE		
	Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
10^9 SRBC + adjuvant	5.08 ±0.04	6.26 ±0.04	6.33 ±0.04	1.94 ±0.17	3.33 ±0.11	3.66 ±0.06
10^9 SRBC	4.22 ±0.04	5.12 ±0.05	5.19 ±0.08	1.32 ±0.09	2.54 ±0.06	2.99 ±0.06
Difference‡	0.86	1.14	1.14	0.62	0.79	0.67
Enhancement§	7 ×	14 ×	14 ×	4 ×	6 ×	5 ×

* Each mean is for 14 or more rats.

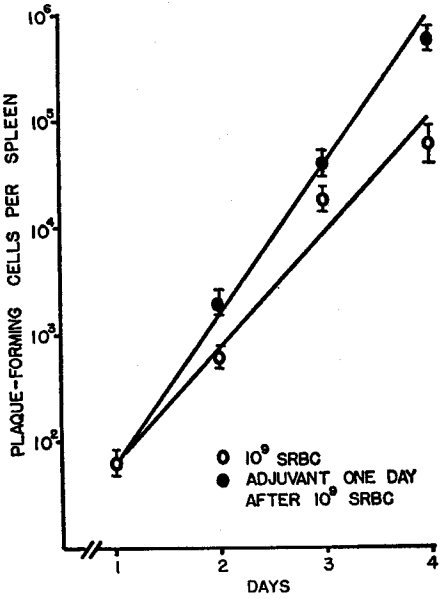
‡ The difference equals the mean for spleen plaque-forming cells or antibody titers of rats immunized with antigen and adjuvant minus the mean for animals immunized with antigen alone.

§ Enhancement equals the antilog of the difference.

In repeated experiments, rats were injected with 5×10^7 or 10^9 sheep erythrocytes and killed 1–5 days after immunization. The results have been combined and plotted on Text-fig. 1. 1 day after immunization, spleen plaque-forming cells were slightly increased above background. Between days 1 and 4, the increase in plaque-forming cells was exponential for both doses of antigen, but the rate of increase was higher for the larger dose of antigen. The average doubling time of plaque-forming cells from days 1 to 4 was 8.3 hr for the lower dose and 6.9 hr for the higher dose of antigen. The number of plaque-forming cells increased slightly from the 4th to the 5th day. The rise in serum titer from days 3 to 5 correlated reasonably well with the rise in plaque-forming cells. All measurable antibody on days 3, 4, and 5 was inactivated by 2-mercaptoethanol.

Responses to Antigen and Adjuvant.—The adjuvant effect of various doses

of pertussis vaccine was measured using 10^9 sheep erythrocytes, i.e., a "saturation" dose of antigen. Doses of 0.4 ml or more of adjuvant produced death or severe weight loss, and therefore were not used. Doses of 0.2 or 0.1 ml of adjuvant produced equal enhancement. Decreasing the dose of adjuvant to 0.01 ml reduced enhancement by about one half. On the basis of these findings, a dose of 0.15 ml of adjuvant was arbitrarily selected as capable of producing maximum enhancement, and this dose was used for all subsequent experiments.



TEXT-FIG. 2. Enhancement of the spleen plaque-forming cell response by adjuvant, 0.15 ml of *B. pertussis* vaccine, given 1 day after immunization with 10^9 sheep erythrocytes. Each point represents the mean of the $\log_{10}$ transformed data ± the standard error of the mean plotted as a vertical bar. The point at day 1 is the mean for 8 rats; each other point is the mean for 6 rats.

Enhancement was equivalent whether this dose of adjuvant was mixed with the antigen in vitro or was injected separately at the time of immunization.

In repeated experiments, rats were injected with 10^9 sheep erythrocytes and 0.15 ml of adjuvant and were killed 1–5 days after immunization. The results have been combined and plotted on Text-fig. 1. 1 day after antigen and adjuvant, spleen plaque-forming cells were slightly increased above background but were equal in number to those detected after immunization with 5×10^7 or 10^9 sheep erythrocytes alone. Between days 1 and 4, the increase in plaque-forming cells was exponential with an average doubling time of 5.2 hr. The number of plaque-forming cells increased slightly from the 4th to the 5th day.

The rise in serum titer from days 3 to 5 correlated well with the increase in plaque-forming cells. Antibody present before the 5th day was inactivated by 2-mercaptoethanol. On the 5th day, low titers of antibody not inactivated by 2-mercaptoethanol were present in the sera. Adjuvant enhancement of the responses to 10^9 sheep erythrocytes is summarized in Table I. Plaque-forming cell responses were enhanced to a greater extent than antibody titers; this discrepancy between the responses will be discussed later.

TABLE II

Colchicine Suppression of Spleen Plaque-Forming Cells on the Third Day of the Primary Response

Immunization	Colchicine treatment*	Plaque-forming cells per spleen‡		
		Day 2	Day 3	Day 4
	hr			
5×10^7 SRBC	None	569 (2.76 ± 0.05)	5,860 (3.77 ± 0.15)	20,790 (4.32 ± 0.07)
	2.5		3,850 (3.58 ± 0.24)	
	5.0		864 (2.94 ± 0.26)	
	7.0		311 (2.49 ± 0.17)	
10^9 SRBC + adjuvant	None	3230 (3.51 ± 0.12)	118,150 (5.07 ± 0.10)	965,470 (5.98 ± 0.12)
	2.5		55,830 (4.75 ± 0.10)	
	5.0		11,640 (4.07 ± 0.26)	
	7.0		7,670 (3.88 ± 0.04)	

* Rats treated with colchicine on the 3rd day after immunization received the drug, 1.3 mg/kg 2.5, 5.0 or 7.0 hr before sacrifice.

‡ The number of plaque-forming cells per spleen is the antilog of the mean for 5 rats; the log of the mean for 5 rats ± the standard error of the mean is given in parentheses.

Controls for experiments using adjuvant included rats injected with 0.15 ml of adjuvant but no sheep erythrocytes. The animals were killed 1–8 days later. Such rats had from 200 to 2000 "background" spleen plaque-forming cells, the greatest increase occurring 3 or 4 days after animals were given adjuvant. By 8 days, spleens again contained usual numbers of background plaque-forming cells. Sera from rats receiving only adjuvant had no measurable antibody to sheep erythrocytes. Adjuvant given alone increased the number of background plaques; however, adjuvant given 1, 2, 3, or 4 days *before* antigen did not enhance the response. In contrast, adjuvant given after antigen caused

enhancement of responses as shown in the following experiment. Each of 44 rats was injected with 10^9 sheep erythrocytes. 24 hr after immunization, 8 rats were sacrificed for spleen plaque-forming cell determinations; 18 rats were injected with 0.15 ml of adjuvant, and the remaining 18 rats served as controls. Groups of 6 rats that received antigen alone and groups of 6 rats that received adjuvant 1 day after immunization were killed 2, 3, and 4 days after immunization. The spleen plaque-forming cell responses are plotted on Text-fig. 2. Adjuvant enhancement was measurable 2 days after immunization, i.e., 1 day after adjuvant was given; the response was exponential from days 1 to 4.

TABLE III

Comparison of Cell Cycle and Cell Doubling Times (See Table II)

Immunization	Colchicine treatment	Cell cycle time*	Mean cell cycle time‡	Mean cell doubling time§
		Day 3	Day 3	Day 2–4
	hr	*hr*	*hr*	*hr*
5×10^7 SRBC	2.5	9.2		
	5.0	6.2	7.7	9.2
	7.0	7.6		
10^9 SRBC + adjuvant	2.5	5.7		
	5.0	5.8	6.4	5.8
	7.0	7.7		

* Cell cycle time is estimated from the extent of colchicine suppression of plaque-forming cells using the method outlined in the Appendix.

‡ Mean of the estimates for the three time intervals.

§ Cell doubling time is estimated from numbers of plaque-forming cells present in the spleens of noncolchicine treated immunized rats on days 2, 3 and 4. Method of calculation is outlined in the Appendix.

Enhancement was almost as great as when adjuvant was given with antigen, as may be seen by comparing the responses of adjuvant treated animals in Text-figs. 1 and 2.

EXPERIMENTAL OBSERVATIONS

A mitotic blocking agent could be used to estimate in vivo cell cycle times of plaque-forming cells providing that the blocking agent did not inhibit release of antibody by cells during interphase, and that cells blocked in mitosis did not release sufficient antibody to be identified. Under these conditions, treatment of immunized animals with a mitotic blocking agent would render plaque-forming cells undetectable only as the cells entered mitosis. The reduction in numbers of plaque-forming cells would be directly proportional to the length of drug treatment, as long as the interval was less than the mean cycle time of

plaque-forming cells. Cell cycle times could be calculated using a mathematical model appropriate for exponentially increasing populations of cells, as described in the Appendix. The following experiments demonstrate the use of mitotic blocking agents for estimating cell cycle times in this way.

Use of Mitotic Blocking Agents to Estimate In Vivo Cell Cycle Times of Plaque-Forming Cells.—Colchicine or Velban given to immunized rats at various times before sacrifice caused reduction in numbers of plaque-forming cells recovered from spleens. The extent of reduction was dependent on the dose of the drug, the method of immunization, and the interval between drug administration and

TABLE IV

Colchicine Suppression of Spleen Plaque-Forming Cells on the Second, Third, and Fourth Days of the Primary Response

Immunization	Colchicine treatment*	Plaque-forming cells per spleen‡		
		Day 2	Day 3	Day 4
	hr			
5×10^7 SRBC	None	825 (2.92 ± 0.11)	2,099 (3.22 ± 0.08)	35,800 (4.55 ± 0.06)
	5	249 (2.40 ± 0.06)	909 (2.96 ± 0.13)	9,952 (4.00 ± 0.19)
10^9 SRBC + adjuvant	None	2971 (3.47 ± 0.11)	113,733 (5.06 ± 0.06)	1,178,995 (6.22 ± 0.06)
	5	296 (2.47 ± 0.21)	8,414 (3.92 ± 0.08)	103,886 (5.02 ± 0.10)

* Rats treated with colchicine received 1.3 mg of drug per kg body weight 5 hr before sacrifice.

‡ The number of plaque-forming cells per spleen is the antilog of the mean for 5 rats; the log of the mean for 5 rats ± the standard error of the mean is given in parentheses.

sacrifice. These parameters of the phenomenon were examined in a large series of experiments. Doses of drugs selected for experiments reported here were: colchicine 1.3 mg/kg or Velban 3.0 mg/kg. The drugs at these doses produced equal effects and could be used interchangeably; reducing either drug dose by one third caused significantly less effect, but increasing the dose 3-fold caused no greater effect than the doses selected. The extent of suppression of spleen plaque-forming cells was dependent on the duration of treatment of animals with the blocking agent. For example, 3 days after immunization with 5×10^7 sheep erythrocytes, five rats received no injections and groups of five rats were injected with Velban, 3 mg/kg, either 4 or 8 hr before sacrifice. The mean numbers of spleen plaque-forming cells were: untreated, 5896 (3.77 ± 0.13);

Velban treatment for 4 hr, 4621 (3.67 ± 0.19); and Velban treatment for 8 hr. 2706 (3.43 ± 0.11).[1]

The extent of drug suppression was also dependent on the immunization procedure as well as the duration of treatment with the blocking agent. For example, 3 days after immunization with 10^9 sheep erythrocytes and adjuvant, five rats received no injections and groups of five rats were injected with Velban, 3.0 mg/kg, either 3 or 5 hr before sacrifice. The mean number of spleen plaque-forming cells were: untreated, 240,932 (5.38 ± 0.06), Velban treatment for 3 hr, 50,100 (4.70 ± 0.06); and Velban treatment for 5 hr, 21,215 (4.33 ± 0.08). In another experiment, 24 rats were immunized with 10^9 sheep erythrocytes; 24 hr after immunization, 12 rats were injected with adjuvant. 3 days after immunization, 6 rats in each group were injected with colchicine, 1.3 mg/kg, 5 hr before sacrifice. Drug treatment reduced the number of spleen plaque-forming cells from 7406 (3.87 ± 0.09) to 3980 (3.60 ± 0.12) in rats immunized with antigen alone, while reduction was from 22,402 (4.35 ± 0.09) to 1456 (3.16 ± 0.12) in rats that received adjuvant 24 hr after immunization. Thus, the extent of suppression produced per hour of drug treatment was greater in animals having more rapid doubling times of spleen plaque-forming cells resulting from immunization with higher antigen doses or adjuvant treatment

TABLE V

Comparison of Cell Cycle and Cell Doubling Times (See Table IV)

Immunization	Colchicine treatment	Cell cycle time*			'Mean Cell Cycle Time‡	Mean cell doubling time§
		Day 2	Day 3	Day 4	Day 2–4	Day 2–4
	hr	*hr*	*hr*	*hr*	*hr*	*hr*
5×10^7 SRBC	5	8.1	10.4	7.7	8.7	8.5
10^9 SRBC + adjuvant	5	5.8	5.6	5.5	5.6	5.2

* Cell cycle time is estimated from the extent of colchicine suppression of plaque-forming cells using the method outlined in the Appendix.

‡ Mean of the estimates for the 3 days.

§ Cell doubling time is estimated from the numbers of plaque-forming cells present in the spleens of noncolchicine treated immunized rats on days 2, 3, and 4. The method of calculation is outlined in the Appendix.

Cycle times measured 3 days after immunization were compared with doubling times of spleen plaque-forming cells in the following experiment. 30 rats were immunized with 5×10^7 sheep erythrocytes; 30 rats were immunized with 10^9 sheep erythrocytes and adjuvant. Five rats in each immunized group were

[1] The numbers in parentheses are means of logarithmically transformed data ± the standard error of the mean.

killed 2, 3, and 4 days after immunization. On the 3rd day, additional rats in each immunized group were injected with colchicine, 1.3 mg/kg, 2.5, 5.0, or 7.0 hr before sacrifice

The results are presented in Tables II and III. The extent of drug suppression was a function of the duration of drug treatment and the immunization procedure. In similarly immunized rats, cycle times were comparable for the various intervals of drug treatment. The mean cycle time of 7.7 hr for plaque-forming cells in animals immunized with the low antigen dose was somewhat shorter than the mean doubling time of 9.2 hr. The mean cycle time of 6.4 hr for spleen plaque-forming cells in animals immunized with the higher dose of antigen and adjuvant was somewhat longer than the mean doubling time of 5.8 hr.

Drug reduction of plaque-forming cells was compared on various days after immunization in the following experiment. 30 rats were immunized with 5×10^7 sheep erythrocytes, and 30 with 10^9 sheep erythrocytes and adjuvant. 10 rats from each group were killed 2, 3, and 4 days after immunization. On each day, the numbers of spleen plaque-forming cells in five animals given colchicine 5 hr before sacrifice were compared with the numbers of spleen plaque-forming cells of five similarly immunized rats that received no colchicine.

The results are presented in Tables IV and V. For similarly immunized rats, drug reduction of numbers of spleen plaque-forming cells was remarkably constant 2, 3, and 4 days after immunization, although numbers of plaque-forming cells increased enormously during this time. Thus, the effect of the drug was independent of the numbers of plaque-forming cells in spleens but was dependent on the method of immunization. Cell cycle times calculated from drug reduction ranged from 8.1 to 10.4 hr in rats immunized with 5×10^7 sheep erythrocytes and 5.5 to 5.8 hr in rats immunized with 10^9 sheep erythrocytes and adjuvant. The mean cycle times and mean doubling times from days 2 to 4 were comparable in similarly immunized animals.

The results for non–drug-treated, similarly immunized rats, presented in Tables II and IV, were combined; mean cell doubling times calculated from the increase in spleen plaque-forming cells occurring between days 2 to 4 are presented in Table VI. Also, in these two experiments, additional rats in both immunized groups were injected with colchicine 5 hr before sacrifice on the 3rd day. Mean cycle times estimated from the combined results are also presented in Table VI. The cell cycle and doubling times are again in good agreement, particularly for animals immunized with 10^9 sheep erythrocytes and adjuvant.

The following observations provide more direct support for the assumption that the drugs reduced the numbers of plaque-forming cells by blocking such cells in metaphase.

Effect of Velban on Mouse Spleen Cells Immunized in Vitro with Sheep Erythro-

cytes.—Dispersed normal spleen cells were cultured in vitro with sheep erythrocytes. The early kinetics and magnitude of the plaque-forming cell response closely parallels that observed in vivo (17, 18). Similar results have been obtained in this laboratory with the additional finding that two functionally different spleen cell populations are apparently required for in vitro induction of the immune response (19). One population consists of larger phagocytic cells or macrophages that adhere to plastic; the second population consists of smaller cells that do not adhere to plastic during short culture periods and are morphologically lymphocytes. The following observations suggest that close physical interaction between macrophages and lymphocytes continues during the in vitro immune response.

TABLE VI

*Comparison of Cell Cycle and Cell Doubling Times**

Immunization	Cell cycle time‡	Cell doubling time§
	hr	*hr*
5×10^7 SRBC	7.4 (5.7–17)	8.8 (7.8–10.2)
10^9 SRBC + adjuvant	5.6 (5.3–6.5)	5.6 (5.1–6.0)

* Selected data from Tables II and IV combined.

‡ The cell cycle time on the 3rd day estimated from the extent of suppression of plaque-forming cells produced by 5 hr of colchicine treatment using the method outlined in the Appendix. The numbers in parentheses represent the approximate 95% confidence limits.

§ Cell doubling times estimated from the numbers of plaque-forming cells in spleens of all similarly immunized non-colchicine-treated rats 2, 3, and 4 days after immunization, using standard methods outlined in the Appendix. The numbers in parentheses represent the 95% confidence limits.

Cultured mouse spleen cells were collected 4 days after in vitro immunization; the harvesting of cultured cells was done with as little mechanical agitation of the cell suspension as possible. Plates for determining numbers of plaque-forming cells and stained smears were prepared from the suspension before and after 30 sec agitation on a vortex mixer. Mechanical agitation of the suspension increased from 4 to 38 the number of plaques produced per 10^6 cells initially cultured. Most plaques produced by nondispersed cells contained a central cluster of cells (Fig. 1); stained smears showed that most of the clusters consisted of numerous lymphocytes surrounding one or more large mononuclear cells. Each plaque produced by the mechanically dispersed cells contained a single centrally located cell. Microscopic examination of suspensions confirmed that mechanical agitation had dispersed the cells; in addition, microscopic examination of cultures before harvesting showed clusters of cells in the culture dishes, indicating that clusters do not result from aggregation of cells during or after harvesting. In three other experiments of similar design, mechanical

dispersion of the cultured cells increased the number of plaques by 9-fold or more, indicating that most plaque-forming cells are in clusters.

Addition of Velban to cultures blocked many cells in metaphase and reduced the number of plaque-forming cells as shown in the following experiments. On the 4th day of culture of in vitro immunized spleen cells, Velban diluted in balanced salt solution was added to give a concentration of 10^{-6} g/ml of culture. The salt solution alone was added to control cultures. Cultures were harvested 6 hr later. Before mechanically dispersing the cells, samples were taken for smears. These samples were treated briefly with hypotonic KCl, fixed in acetic acid-ethanol, spread on slides in 55% methanol/45% acetic acid, air-dried, and stained. The remainder of the sample were mechanically dispersed and plates

TABLE VII

Cycle Times for Plaque-Forming Cells of In Vitro Immunized Mouse Spleen Cells

Velban treatment*	Exp. 1		Exp. 2		Exp. 3	
	Plaque-forming cells‡	Cell cycle time§	Plaque-forming cells‡	Cell cycle time§	Plaque-forming cells‡	Cell cycle time§
hr		*hr*		*hr*		*hr*
None	65	—	41	—	132	—
3	35	8.8	20	7.6	82	7.4
6	16	8.0	7	7.1	15	7.1

* Cultures, 4 days after in vitro immunization, were treated with Velban 3 or 6 hr before harvesting.

‡ Plaque-forming cells are recorded as the number per 10^6 spleen cells initially cultured.

§ The cell cycle time was calculated using the model presented in the Appendix. The corresponding doubling times calculated from the increase in plaque-forming cells from days 2 to 4 for Experiments 1 to 3 were 7.5, 8.4, and 6.5 hr respectively.

were prepared to determine the total numbers of plaque-forming cells in drug-treated and control cultures. In non–Velban-treated cultures, about 1% of cells were in metaphase; the per cent of cells in metaphase was not discernibly greater in clusters of cells. In marked contrast, about 10% of all cells in cultures treated with Velban for 6 hr were in metaphase, and 20–80% of cells in clusters were in metaphase. Fig. 2 shows a small cluster of 11 cells, 4 of which are in metaphase. Velban treatment for 6 hr reduced from 61 to 13 the total number of plaque-forming cells per 10^6 spleen cells initially cultured. The cell cycle time based on Velban suppression was 8.2 hr.

In three other experiments, cultures were treated with Velban 4 days after in vitro immunization. The numbers of plaque-forming cells in cultures treated with the drug for 3 or 6 hr were compared with the numbers in control cultures. The reduction in plaque-forming cells was directly proportional to the duration of drug treatment, Table VII. Cell cycle times calculated from the results of

Velban treatment for 3 or 6 hr differed only slightly, and the cycle times corresponded quite well with doubling times calculated from the exponential increase in plaque-forming cells (Table VII).

DISCUSSION

The use of Velban or colchicine to estimate cell cycle times in these experiments was based on the premise that cells blocked in mitosis do not release sufficient antibody to be identified, but that release of antibody by cells in interphase is not affected by the drugs. The first assumption is consistent with observations that amino acid incorporation into protein is markedly decreased in other types of cells blocked in metaphase by colchicine (20), possibly as a result of disaggregation of polyribosomes (21). Many observers have found occasional (<1%) cells in the centers of plaques in mitosis, but mitosis may have occurred in culture after release of antibody, as has been reported recently (13). However, we have observed no plaque-forming cells in mitosis when cells were obtained from animals treated with Velban or colchicine, though cells in mitosis were evident in stained smears of spleen cells obtained from such animals. Several observations support the second assumption, i.e., that neither drug affects release of antibody by cells in interphase. The distribution of size of plaques produced by spleen cells from immunized rats was the same whether the cells were obtained from rats treated with Velban, or colchicine, or were from untreated rats. Presumably, the drugs did not produce their effects by causing progressive diminution of antibody synthesis and by this mechanism cause reduction in numbers of detectable plaque-forming cells. Very large doses of Velban or colchicine given 10–15 min before sacrifice or very high concentrations of the drugs added to the culture medium did not reduce numbers of plaque-forming cells, indicating that the drugs are not directly toxic to these cells. The extent of reduction was directly proportional to the duration of drug treatment, so long as the interval between drug administration and animal sacrifice was less than the calculated cell cycle time. The drug effect was independent of the number of plaque-forming cells in spleens. It seems more reasonable to assume, therefore, that these findings are the result of the well-known action of colchicine or Velban to arrest cells in metaphase, than to assume that these drugs have some other time-dependent, antigen-dose–dependent toxicity for plaque-forming cells.

Velban has been used to measure cell cycle times of tumor cells in a model very similar to the one described here (22). Inhibition of plating efficiency of tumor cells was proportional to the duration of treatment with the drug; cell cycle times estimated by this method agreed well with in vivo methods. It is of interest that in this model, Velban treatment of cells for a complete estimated cell cycle time abolished the capacity of only 90–95% of tumor cells to grow in culture. In repeated experiments, drug reduction of plaque-forming cells never

exceeded 95%. It is not known why Velban or colchicine treatment for an estimated cell cycle time or longer does not abolish all plaques. The calculated cycle times represent the mean of a range of cycle times; the frequency distribution of cycle times has not been determined. The 5–10% of cells surviving the effects of the drug for the length of a mean cycle time may represent the tail of distribution of cycle times, which could have a marked degree of skewness. In any event, the tumor cell model indicates that the phenomenon is not peculiar to antibody-forming cells.

Using a hot pulse labeling technique with spleen cell cultures immunized in vitro, Dutton and Mishell concluded that essentially all antibody-forming cells appearing in cultures could arise by cell division (23). We arrive at the same conclusion using Velban in spleen cell cultures immunized in vitro. Most plaque-forming cells in culture are aggregated in clusters. In cultures treated with Velban for 6 hr, many of the cells in clusters were in metaphase; also, Velban caused a marked reduction in the total number of plaque-forming cells. These observations support the premise that the drug caused reduction of plaque-forming cells by arresting these cells in metaphase.

It might be argued that plaque-forming cells arise from an exponential rate of conversion from some other population of cells which had not previously produced antibody. In such a model, it might be expected that mitotic blocking agents would interfere with the continued conversion of cells solely by preventing multiplication of the precursor cells. Plaque-forming cells already present would not be affected by the mitotic blocking agents. Even if all plaque-forming cells arose by such conversion from a cell population with a cell cycle time as short as 5 hr the maximum possible suppression by treatment with a mitotic blocking agent for 5 hr would be 50%. The data presented here are not consistent with such a model.

Recruitment or continued activation of potential antibody-producing cells was assumed to have occurred during the early primary immune response, because generation times as short as 5.4 or 6.1 hr had not been reported for immunocompetent or other mammalian cells (14). This general argument for recruitment of cells, based solely on an assumed maximum rate for division of mammalian cells, is invalidated by observations that one population of lymphocytes in calf thoracic duct lymph has a cell cycle time of 5.5–6.0 hr (24, 25).

Adjuvants might cause recruitment of additional responding cells, but the present findings provide no support for this possibility. The number of spleen plaque-forming cells is equivalent 24 hr after immunization whether animals were immunized with antigen alone or antigen and adjuvant. When adjuvant was given 1 day after immunization, enhancement 2, 3, and 4 days after immunization was almost as great as when adjuvant was given at the time of immunization; thus, enhancement cannot be accounted for by assuming appreciable recruitment of additional responding cells during the first 24 hr after

immunization. If appreciable recruitment of cells occurred more than 1 day after adjuvant was given, this would result in an increased slope of the $\log_{10}$ normal plot of the plaque-forming cell response beginning when additional cells entered the population of already responding cells. No such upward inflexions have been observed.

It might be argued that the plaque-forming cell technique measures, at a point in time, the number of cells capable of synthesizing and releasing sufficient antibody to produce detectable plaques of lysis. If adjuvant had the effect of increasing the amount of antibody synthesized or released by cells, then this effect would result in increased numbers of cells that could be enumerated by the plaque-forming cell technique. Adjuvant enhanced the plaque-forming cell response to a greater extent than it enhanced serum antibody titers. This discrepancy between adjuvant enhancement of the responses might be accounted for if the amount of antibody synthesized by a cell in vivo is proportional to the length of interphase. Possibly, cells dividing every 5 hr synthesize less antibody during each cell generation than cells dividing every 7 or 9 hr. In any event, the data do not support the suggestion that individual cells from adjuvant treated animals synthesize more antibody.

The increased responses produced by increased doses of antigen or adjuvant are apparently the result of increased rates of division of responding cells. This is indicated not only by steeper slopes of $\log_{10}$ normal plots of responses, but also by shorter cell cycle times obtained when mitotic blocking agents are used. Admittedly, our experiments are not sufficiently accurate to exclude recruitment of a few additional responding cells, either as a component of the continued response to a single dose of antigen or as part of the enhanced response with adjuvant. Our findings, however, are definitely contrary to those of Sterzl and Tannenberg who concluded on the basis of radioautographic studies that spleen plaque-forming cells arose in large part from cells that had not undergone recent mitosis (9, 11, 12). We believe there are technical and conceptual difficulties with these kinds of radioautographic studies; additional work will be necessary to resolve inconsistencies between these and the present studies.

Cell doubling times during the early primary response are equivalent by the two methods used here. After this phase, and during the secondary response, the kinetics are more complex, first because of the appearance of cells releasing "7S" hemolysin and second because the numbers of plaque-forming cells decrease. The decrease may be due to either loss of cells from the spleens by death or emigration, or by change in function of cells. Mitotic blocking agents may provide a simple means for obtaining useful information on the kinetics of cell responses during the late primary and secondary responses; however, the model as presented here, cannot be applied directly to such studies without considering these parameters.

The results are consistent with a cell selection theory of antibody formation (26–29). Antigenic stimulation causes cells to proliferate and to synthesize antibody; the magnitude of the response is dependent primarily on the rate of division of initial responding antibody-producing cells and their progeny. The rate of division of antibody-forming cells might be determined by the rate of interaction between these cells and macrophages that have phagocytized antigen. In the present studies, lymphoid cells, some of which are undoubtedly plaque-forming cells, were found clustered around macrophages. Anatomical connections between lymphocytes and macrophages have been described (30, 31). Higher antigen doses or an adjuvant might enhance the rate of interaction between the two different cell types and by this effect increase the rate of division of antibody-forming cells.

SUMMARY

Mitotic blocking agents, colchicine or Velban, were used to estimate cycle times of spleen cells which release hemolysin for sheep erythrocytes (plaque-forming cells). The cells were obtained either from rats immunized with sheep erythrocytes or from cultures of mouse spleen cells immunized in vitro with the same antigen.

2, 3, or 4 days after immunization, animals or cell cultures were treated with mitotic blocking agents for periods of time ranging from 2.5 to 7 hr; plaque-forming cells were then enumerated. Decreased numbers of plaque-forming cells were found after such treatment. The extent of reduction was a function of duration of the drug treatment and the method of immunization, but was independent of the time after immunization. The evidence presented is consistent with premises that: (*a*) plaque-forming cells in mitosis do not release sufficient antibody to be detected, (*b*) mitotic blocking agents, by arresting plaque-forming cells in metaphase, prevent not only detection of these cells but also the increase in number of plaque-forming cells which would have resulted from cell division, (*c*) mitotic blocking agents do not affect release of antibody by cells in interphase.

Cell cycle times, based on the extent of reduction of plaque-forming cells per unit time of drug treatment, were estimated using a mathematical model appropriate for an exponentially increasing population of cells. Cell cycle times estimated using the mitotic blocking agents agreed well with cell doubling times calculated from the increase in plaque-forming cells occurring 1–4 days after immunization. Increased responses produced by higher antigen doses or treatment of immunized animals with an adjuvant resulted from an increased rate of division of responding cells and their progeny.

The results are consistent with a cell selection theory of antibody formation. Antigenic stimulation causes relatively few cells to proliferate and to synthesize antibody; apparently the magnitude of the response is dependent primarily

on the rate of division of responding cells. It is suggested on the basis of observations of in vitro–immunized cell cultures that the rate of division of responding cells may be dependent on the rate of interaction between two cell types, both of which are essential for the in vitro plaque-forming cell response.

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APPENDIX

In the body of the paper, a distinction is made between cycle time and doubling time. Doubling time is calculated from the increase in the spleen plaque-forming cells occurring 1–4 days after immunization; it is the time required for the population to double. It is assumed that the number of plaques formed follows an equation of the form

$$y(t) = ke^{bt}$$

where k is a constant for each experiment, e is the Naperian constant, b is the logarithm of 2 divided by the doubling time, and t is time from the beginning of the exponential response.

Taking logarithms

$$\ln y(t) = \ln k + b \ln t,$$

the logarithms of the observed number of plaques are assumed to be normally distributed. Thus, the situation is that of simple regression analysis. Standard bounds can be calculated for the doubling time from the variance of the slope (34).

The cell cycle time is estimated from the reduction in numbers of observable spleen plaque-forming cells produced by mitotic blocking agents. A mathematical model was constructed to estimate cell cycle times and to determine confidence limits of the estimates. The model was constructed from the following postulates:

(*a*) The antibody-forming cell population is increasing exponentially.

(*b*) These cells are completely asynchronous with respect to stage of the mitotic cycles.

(*c*) All cells have a fixed cycle time, c, which is the interval between successive mitoses.

(*d*) All cells in mitosis at the moment of administration of the mitotic inhibitor become arrested in metaphase. (All cells that subsequently enter mitosis are also arrested in metaphase.)

(*e*) All antibody-producing cells in mitosis, whether arrested in metaphase or not, do not form observable plaques.

(*f*) There is a sharply defined point somewhere at the beginning of mitosis when cells no longer produce a plaque and a point near the end of mitosis when daughter cells again produce observable plaques. The time that the cells do not produce plaques corresponds roughly to the mitotic time and is called a.

(*g*) To make analysis tractable, it is assumed that a cell doubles immediately upon entry to mitosis. This assumption does not affect the analysis since cells in mitosis are unobservable.

Let $z(t)$ be the total number of cells present at time t. Postulate (*a*) implies that

$$z(t) = ke^{bt} \tag{1}$$

for some positive constants k and b. Postulate (*c*) implies that the number of cells present doubles in time c, thus $e^{bc} = 2$ or $b = \ln 2/c$. The constant k represents the number of cells present at the time that the response to immunization begins.

By postulates (*a*) and (*g*), the number (m) of cells entering mitosis between times t_1 and t_2, ($t_1 < t_2$) is the same as the number of cells added during this interval, i.e.,

$$m(t_1, t_2) = z(t_2) - z(t_1) \tag{2}$$

The number of observable plaque-forming cells at time t is the total number of cells less those in mitosis. The number of cells in mitosis will be twice the number which entered mitosis in the interval $t - a$ to t. Thus, the number of cells observable at time t will be

$$y(t) = z(t) - 2m(t - a, t) = 2z(t - a) - z(t) = ke^{bt}(2e^{-ba} - 1) \tag{3}$$

Assuming that a mitotic blocking agent is injected x hr prior to observation, the effect is to render unobservable all cells in mitosis at the time of injection [i.e., $2m(t - x - a, t - x)$ cells] and all cells entering mitosis thereafter. Thus, twice the number of cells which entered mitosis in the interval $t - x - a$ to t are unobservable. Let $w(t, x)$ be the number of observable cells at time t when mitotic inhibitor was injected x hr prior to t (where $x < c - a$ to avoid negative numbers being predicted). Then,

$$w(t, x) = z(t) - 2m(t - x - a, t) = 2z(t - x - a) - z(t) = ke^{bt}(2e^{-b(x+a)} - 1) \quad (4)$$

Inspection of the data showed that the scatter about the mean in the experimental groups was grossly dependent on the mean. Logarithmic transformation adequately corrected this and was used throughout these calculations to achieve homoscedasticity.

Having made this transformation, it is assumed that the data are drawn from Gaussian distributions and that these distributions have means given by ln $y(t)$ or ln $w(t, x)$ and common variance.

To estimate the parameters, b and a, likelihood methods originated largely by Fisher (32) were used. These methods give the probability density of the possible outcomes of the experiment, given the parameters of the model, namely a, c, k, and the variance of the above-mentioned Gaussian distributions.

The principle of maximum likelihood is to take as estimates of the parameters those values which maximize the probability density at the point representing the actual outcome of the experiment. Fortunately, it is a property of this model that for fixed values of a and c, it is easy to compute the values of the other parameters that maximize the likelihood. (Details will be made available upon request.) A tabulation can be prepared of this likelihood maximized over these parameters against a and c and the maximum obtained.

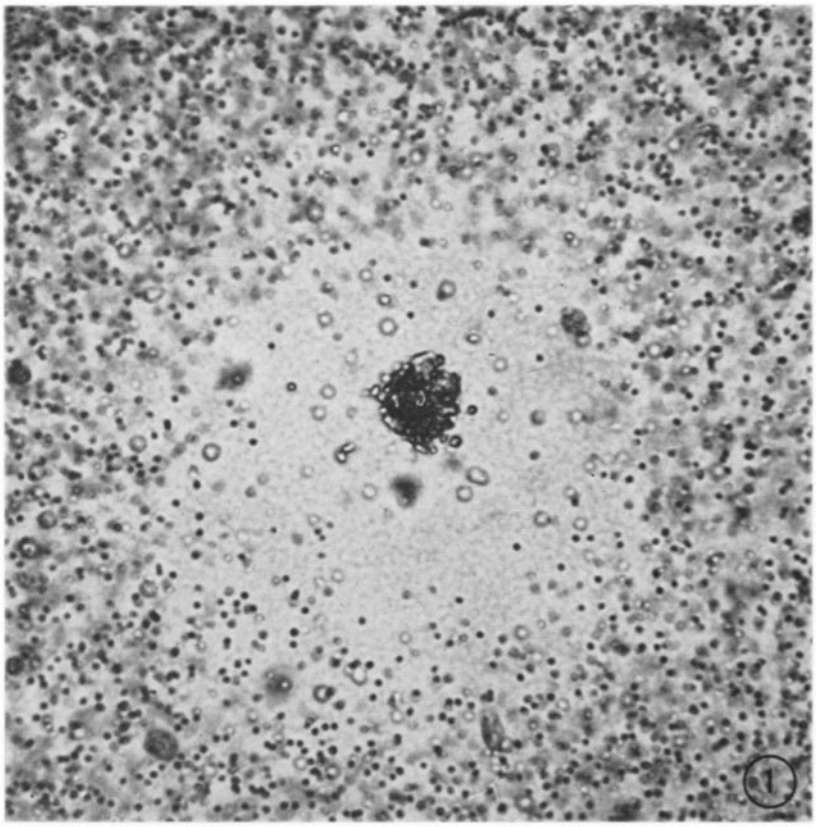
Asymptotically, twice the negative logarithm of the ratio of the likelihood at a nonoptimal parameter point to the maximum likelihood is distributed as χ^2 with the degrees of freedom equal to the number of estimated parameters (four in this instance). Thus, it is possible to set approximate confidence limits on the estimates. A recent exposition of these methods is presented by Rao (33).

The likelihood function changed very little for different values of c and a if $c - a$ remained the same. Hence, $c - a$ is the quantity being estimated in these experiments, given this model. Therefore, the results for $a = 0$ are those reported in the paper. It is possible to substitute appropriate estimates for a. Reported estimate for mitotic time of lymphoid cells range from 15 to 45 min (25, 31). Estimates and confidence limits may be increased by this amount.

EXPLANATION OF PLATES

PLATE 101

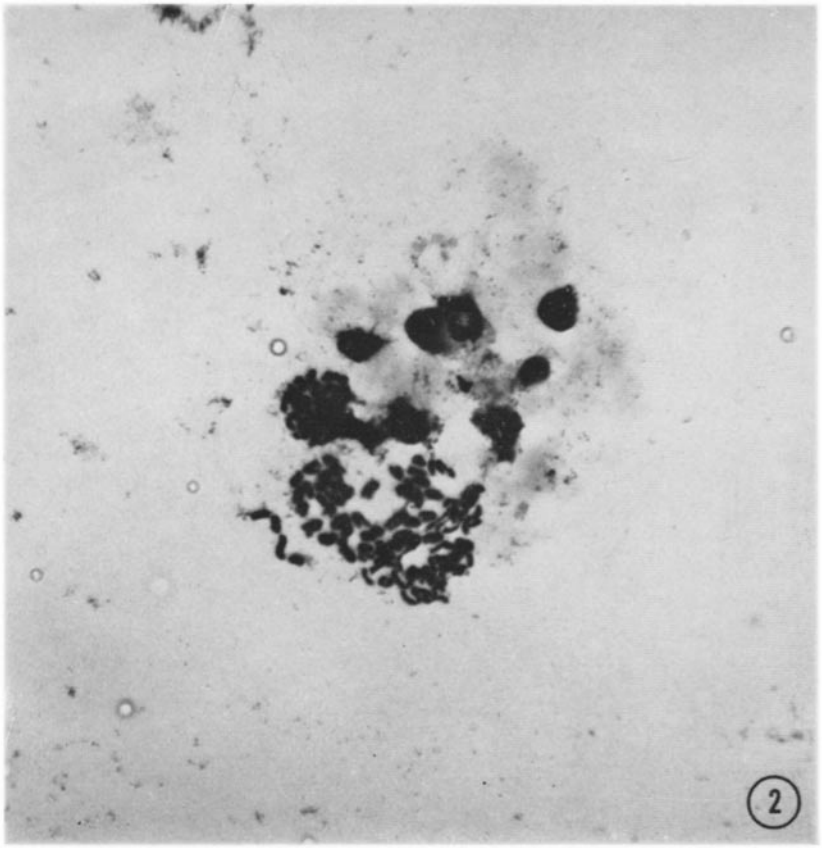
FIG. 1. A hemolytic plaque produced by a cluster of cells from a culture of mouse spleen 4 days after in vitro immunization with sheep erythrocytes. × 200.



(Rowley et al.: Antibody-forming cells and early immune response)

PLATE 102

FIG. 2. A small cluster of cells from a culture of mouse spleen cells 4 days after in vitro immunization with sheep erythrocytes. Velban was added to the culture 6 hr before harvesting. 4 of 11 cells are in metaphase. × 730.



(Rowley et al.: Antibody-forming cells and early immune response)