RADIOAUTOGRAPHIC STUDIES OF PLAQUE-FORMING CELLS

I. Antigen-Stimulated Proliferation of Plaque-Forming Cells*

By AURELIA M. C. KOROS, Ph.D., JOHN M. MAZUR,‡ and MARGARET J. MOWERY

(From the Department of Microbiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213)

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In a primary immunological response in vivo, antigen seems to stimulate immunologically competent cells to proliferate and to produce progeny cells which can make specific antibody. It has been suggested that the dose of antigen regulates the rate of division of competent cells (1–4),[1] but the evidence for this has not been conclusive.

The present study provides evidence of proliferation of plaque-forming cells. Spleen cells from mice immunized with different doses of sheep erythrocytes were pulse-labeled in vitro with tritiated thymidine and analyzed by a radioautographic modification of the Jerne-Nordin (5) plaque technique. Spleens from mice inoculated with high doses of antigenic sheep red cells yielded higher percentages of labeled plaque-forming cells than spleens from mice inoculated with low doses of antigen. The results support the hypothesis that antigen stimulates specific immunocompetent cells to divide and that the rate of division is dependent on the dose of antigen.

Materials and Methods

Mice.—Inbred AKR male mice 10–17 wk old from Jackson Laboratories, Bar Harbor, Maine, were used in all experiments. Mice of the same shipment and age were used in individual experiments.

Reagents.—Powdered Eagle's medium with L-glutamine but without $NaHCO_3$ was obtained from Grand Island Biological Laboratories, Grand Island, N. Y. Distilled water and $NaHCO_3$ (0.7 g/liter) were added to prepare twice concentrated medium which was filtered through an S1 Seitz sterilizing filter and stored at 4°C. Sheep blood in Alsever's solution was obtained from either Grand Island Biological Labs or Baltimore Biological Laboratories, Baltimore, Md. Fresh frozen guinea pig serum obtained from Hyland Laboratories, Los Angeles

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[1] Jerne, N., A. Nordin, C. Henry, H. Fuji, and A. Koros. 1965. The agar plaque technique, the target of the antigen and clonal selection. Personal communication to IEG No 5, Scientific Memo No. 46.

Calif., diluted 1/10, was used as a source of complement. Bacto-Agar from Difco Laboratories, Detroit, Mich., was prepared either as a 1.4% or 3% solution by autoclaving a suspension of agar in distilled water on the day of use. These twice concentrated solutions were kept in a 45°C water bath and diluted with equal parts of twice concentrated Eagle's medium (also at 45°C) immediately before use. DEAE-dextran, having a molecular weight of approximately 2×10^6, was obtained from Pharmacia, Uppsala, Sweden. Stock solutions of 10 mg/ml in Eagle's medium were stored at −10°C. Tritiated thymidine having a specific activity > 10C/mM was obtained in 70% ethanol: water solution (No. NET 027E) from New England Nuclear Corp., Boston, Mass. A fresh dilution of isotope was made for each experiment as follows: 3.8 ml of Eagle's medium was placed in a 25 ml plastic screw cap vial (Packard Instrument Co., Downers Grove, Ill.); 0.2 ml of a 1 mc/ml solution of isotope was added under the surface of the Eagle's medium by means of a sterile disposable plastic pipet; 0.4 ml of this solution which contained 20 μc was used for each incubation tube (see below). Giemsa blood stain (azure B type) was diluted 1/50 with buffered water immediately before use. NTB3 nuclear track emulsion, D-11 developer, acid fixer, and Wratten series 1 Safelight filters were all obtained from Eastman Kodak Co., Rochester, N. Y. Emulsion was stored at 4°C. Developer and fixer were prepared 1–7 days before use, stored at 4°C, and solutions were brought to room temperature immediately before use.

Preparation of Sheep Red Cells for Plating.—Blood cells were aged at 4°C for 6 to 10 days before being used for plating. Immediately before use the cells were centrifuged at 300 *g* at 4°C. The buffy coat was removed with a sterile pipet and the remaining red cells were resuspended in at least 10 volumes of phosphate-buffered saline. This procedure was repeated three times and cells were finally suspended to 20% for use in plating on base layers (5–7) or 50% for use in thin layers (see below). Buffy coat cells were removed routinely from sheep cell suspensions to be used for thin layers to eliminate the rare appearance of false plaques caused by sheep granulocytes (7).

Preparation of Sheep Red Cells for Injection.—Sheep cells were used upon arrival or after storage for 6 to 10 days at 4°C. Cells were washed three times as described above, but buffy coat cells were not removed. A final 20% suspension was made which contained approximately 4×10^9 cells/ml. Further dilutions were made as needed, counted in a Levy counting chamber, and injected into mice.

Immunization.—Mice were inoculated intravenously via a lateral tail vein with 0.1 ml of a 20% suspension of sheep red cells or 0.1 ml of appropriate dilutions thereof. 0.1 ml of a 20% suspension contained approximately 4×10^8 sheep red cells (Table I). The actual numbers of sheep red cells were counted for each experiment except Experiment IV (Table I).

Preparation of Agar Base Layers.—Base layers containing 1.5% agar in Eagle's medium were prepared in 85 x 15 mm disposable Petri dishes 2–16 days before each experiment as described previously (6, 7).

Preparation of Spleen Cell Suspensions.—Groups of from 3 to 8 mice (Table II) were etherized and exsanguinated by cardiac puncture in the same order in which they had been injected. Each spleen was removed and processed aseptically throughout. Groups of spleens from mice inoculated with the same dose of sheep red cells were collected in an 85 mm glass Petri dish containing about 10 ml Eagle's medium and a circular wire grid (stainless steel cloth, type 304 C x 60, Small Parts Inc., Miami, Fla.) held between two metal rings. Spleens were kept on ice until all mice had been sacrificed. The spleens were then minced with scissors and pressed through the wire mesh with a rubber policeman. To remove connective tissue and any remaining cell clumps, cell suspensions were filtered through two layers of cheesecloth (Curity No. 90, Kendall Co., New York, N. Y.) into a graduated 50 ml screw cap centrifuge tube. The total cell volume was adjusted with Eagle's medium to give one spleen equivalent per 5 ml. Aliquots were removed for subsequent incubation with isotope, for cell counting, and for plating on base layers.

TABLE I

Examples of Variables Involved in Six Separate Experiments

Experiment No.	Age of mice	Nos. of sheep red cells injected as 0.1 ml of a 20% suspension	Time of sacrifice	Sequence of plating cell suspensions	Specific activity of tritiated thymidine	Time emulsion exposed
	wk		*hr*		*curies/mM*	*days*
IV	10	not counted	72 approx.	4×10^8* (15 min → 120 min)	13.7	7
V	10	3.9×10^8	72	$4 \times 10^8 \rightarrow 4 \times 10^5$	13.7	7
VIII‡	14	3.9×10^8	73	$4 \times 10^8 \rightarrow 4 \times 10^5$	12.5	5
IX	16	4.8×10^8	72	$4 \times 10^8 \rightarrow 4 \times 10^5$	12.5	7
X	17	3.6×10^8	67	$4 \times 10^5 \rightarrow 4 \times 10^8$	12.5	6
XI	10	3.7×10^8	68	$4 \times 10^5 \rightarrow 4 \times 10^8$	17.4	5

* Spleen cells pooled from 5 mice; aliquots containing 1 spleen equivalent per tube were incubated with 20 μc of tritiated thymidine as described in text; samples were plated at 15 min, 30 min, 60 min, 120 min.

‡ Thin layers incubated and plaques formed as described in Materials and Methods at 37°C in the presence of 5% CO_2 and 95% air. All other experiments were performed in the absence of CO_2.

Counting of Spleen Cell Suspensions.—Aliquots containing about 10^7 cells/ml were diluted further in a solution of 0.1% methyl-rosaniline chloride in 0.1 M citric acid and counted in a Levy counting chamber.

Pulse Labeling of Spleen Cells.—Spleen cell suspension aliquots, each containing one spleen equivalent per 5 ml, were dispensed into graduated conical 15 ml centrifuge tubes with siliconed rubber stoppers. Cells were centrifuged for 5 min at 150 *g* at 4°C and concentrated to 1 ml. The cells were resuspended by mixing on a vortex mixer, and all tubes were kept on ice until after isotope had been added. 20 μc of tritiated thymidine having a specific activity of at least 12.5 C/mM (Table I) in a volume of 0.4 ml were added per tube with a disposable plastic 1 ml pipet. A new pipet was used for each tube and care was taken to deliver all isotope under the surface of the liquid. After the addition of isotope, the entire rack of tubes was placed in a 37°C water bath for 30 min. Tubes were shaken frequently and periodically mixed on the vortex mixer during the incubation. At the end of 30 min incubation, 10 ml of Eagle's medium at 4°C were added to prevent further incorporation of isotope; the tubes were immediately immersed in an ice bath, and then were centrifuged 5 min at 150 *g* at 4°C; supernatant was removed and the cells resuspended in Eagle's medium to the concentration desired for plating. Suspensions were usually prepared to contain approximately 10^7 cells/ml and 0.1 ml was used for each plate.

Plating of Labeled Cells in Thin Layers.—A modification of the technique of Jerne et al. (6, 7) was used to permit radioautographic analyses of large numbers of plaque-forming cells (3, 7). The fact that mice inoculated with low doses of sheep red cells have very few plaque-forming cells in their spleens 3 days after immunization (1–2)[1] presented a special problem that required the preparation of many plates, although few plaque-forming cells could be recovered. In order to survey such large populations of spleen cells, which contained only a few plaque-forming cells, under conditions providing for the occurrence of only a single cell in a plaque, about 200 plates had to be prepared for each experiment. The actual plating of the cells required about 4 hr during which time cell suspensions to be plated were kept in an ice bath. Care was taken to vary the sequence of plating the different cell suspensions in each experiment

TABLE II

Tritiated Thymidine-Labeled Plaque-Forming and Non-Plaque-Forming Spleen Cells from Mice Injected with Different Doses of Antigenic Sheep Red Cells‡*

Experiment No.	Dose of sheep red cells injected	No. of mice per group	Plaque-forming cells			Non-plaque-forming cells		
			Total counted	Labeled	Per cent labeled	Total counted	Labeled	Per cent labeled
V	4×10^8	3	211	121	57.3	1324	28	2.1
	4×10^7	3	70	40	57.1	1075	16	1.5
	4×10^6	3	36	13	36.1	2807	49	1.7
	4×10^5	3	17	3	17.6	4194	101	2.4
VIII	4×10^8	3	370	187	50.5	1127	50	4.4
	4×10^7	3	61	25	41.0	1673	58	3.5
	4×10^6	3	25	3	12.0	3037	156	5.1
	4×10^5	4	28	1	3.6	2470	90	3.6
IX	4×10^8	4	276	155	56.2	1104	64	5.8
	4×10^7	4	86	41	47.7	1724	82	4.8
	4×10^6	4	24	6	25.0	3440	174	5.1
	4×10^5	4	11	1	9.1	2796	415	14.9
X	4×10^8	4	143	74	51.7	1255	68	5.4
	4×10^7	4	41	26	63.4	1747	87	5.0
	4×10^6	8	42	6	14.3	4827	195	4.0
	4×10^5	8	7	0	0	1272	29	2.3
XI	4×10^8	5	148	93	62.8	1025	31	3.0
	4×10^7	3	112	67	59.8	1420	50	3.5
	4×10^6	8	129	71	55.0	6714	130	1.9
	4×10^5	8	20	4	20.0	3045	56	1.8

* Spleen cells pulse-labeled in vitro 30 min at 37°C with 20 μc of tritiated thymidine.

‡ Mice sacrificed 3 days after a single intravenous injection of sheep red cells.

(Table I) to avoid a possible systematic error resulting from the long delay between the first sample plated and the last.

Immediately before plating the cell suspensions, equal parts of a 1.4% agar solution and twice concentrated Eagle's medium were combined at 45°C, usually in batches of 100 ml. DEAE-dextran was added to this mixture to give a final concentration of 0.5 mg DEAE-dextran/ml. 2.0 ml aliquots of the 45°C agar mixture were dispensed into 100 x 13 mm disposable glass tubes kept in a rack in a 45°C water bath. 0.1 ml of 50% sheep red cells and 0.1 or 0.2 ml of spleen cell suspension were added in rapid succession to a single tube; the contents of the entire tube were then poured onto an empty disposable plastic Petri dish (Optilux 85 x 15 mm, No. 1001, Falcon Plastics, Los Angeles, Calif.). The agar mixture was spread uniformly over the surface of the Petri dish by rapidly tilting the dish from side to side. Excess agar was immediately shaken off the dish into a large pan by four rapid shakes of the wrist. About $\frac{1}{5}$ of agar cell mixture adhered to the plastic dish as determined by weight and by plaque count (7). The thin layer was about 70 μ thick (7) in its wet state. Each tube was prepared and plated

individually. The thin layers were flooded with about 10 ml Eagle's medium, and as the dishes were prepared they were covered with lids and incubated for 1 hr at 37°C. The Eagle's medium overlay was then poured off, and 3 ml of complement was added to each dish. Dishes were incubated an additional ½ hr at 37°C at which time plaques could be detected grossly. Complement was poured off; each dish was drained thoroughly and placed without a lid on a tray for drying.

Preparation of Radioautographs.—Plates drained of complement were rapidly fan-dried in a 37°C room for about 1 hr. The thoroughly dried plates were covered with about 20 ml of 95% ethanol and fixed overnight (approximately 8 hr). Alcohol was poured off and plates were air-dried at room temperature. Plates could be stored in this state to await further processing or they could be processed immediately.

Plates were subsequently covered with about 10 ml of cold 5% trichloroacetic acid (TCA) in a 4°C cold room for 5 min to remove any acid-soluble intracellular radioactive material. TCA was poured off and 70% ethanol was added to each dish. Dishes were then returned to the laboratory and remaining TCA was removed by a series of six changes of 70% ethanol every ½ hr at room temperature. Plates were then thoroughly air-dried; the agar layer in this dried state was about 2 μ thick (7). Plates could be stored at this stage to await further processing or they could be processed immediately.

Subsequent steps were performed in a photographic darkroom using Wratten series 1 Safelight filters. NTB3 nuclear track emulsion was thawed in a 40°C water bath. A single lot of emulsion was used for each experiment. Each Petri dish containing the dried agar cell layer was rinsed with distilled water prior to being coated, to facilitate the spreading of emulsion; the water was shaken off, and about 2 ml of emulsion was added to a single dish by means of a disposable Pasteur pipet. The emulsion was spread rapidly by tilting the dish from side to side, and excess emulsion was immediately shaken off the dish by four rapid shakes of the wrist. Each dish was coated individually and all plates were dried at room temperature in the darkroom for 2–3 hr. Lids were then replaced and the plates were inverted and packaged in stainless steel Petri dish containers. Each container was filled with 14 dishes and a gauze bag containing drierite (anhydrous $CaSO_4$). The containers were sealed with tape, wrapped in aluminum foil and stored at 4°C for 5–7 days to allow for adequate exposure of the emulsion.

Plates were processed further under Wratten series 1 filters at room temperature which varied from 20° to 24°C between experiments. About 10 ml of D-11 developer was poured onto each plate and left for 2 min. Each plate was rinsed once in tap water; about 20 ml of fixer was poured onto each plate and left for 5 min. Plates were next washed for 20 min in running tap water, rinsed five times in distilled water, and air-dried.

Staining of Cells in Radioautographic Preparations.—Plates were covered with Giemsa stain for 8 min, rinsed five times in distilled water, and air-dried. Plaques were visible grossly. A circle was drawn with waterproof ink around each plaque from the under surface of the Petri dish by a single observer. The rims were cut off the Petri dishes; a tracing was made of each plate and circles numbered to allow for identification of plaques.

Inspection of Plates for Plaque-Forming and Non-Plaque-Forming Cells.—All circled areas were examined under 100 × magnification and plaques located. Three observers compiled data from coded plates of five separate experiments. Because plaques differ in size and degree of clarity (7, 8), only those plaques which could be classified unquestionably by the three observers were used in the present study. The central cell in each plaque was examined under oil immersion at 1000 × magnification. Plaque-forming cells were initially scored as being labeled or unlabeled.

Each plate was systematically scanned from left to right under 1000 × magnification and any spleen cell not occurring inside a plaque was classified as labeled or unlabeled. Approximately 200 non-plaque-forming cells were inspected per plate in most experiments, e.g., Table III.

TABLE III

*Recovery of Plaque-Forming Cells in Radioautographic Preparations from Experiment No. V**

Dose of sheep red cells injected	Plate No.	Plaque-forming cells			Non-plaque-forming cells		
		Total plaques recovered	Labeled	Per cent labeled	Total cells counted	Labeled	Per cent labeled
4×10^8	21	51	27	52.9	214	2	0.94
	22	46	26	56.5	221	5	2.3
	23	38	20	52.6	226	4	1.8
	24	34	23	67.6	239	9	3.8
	25	42	25	59.5	210	3	1.4
	26	Not counted	—	—	214	5	2.3
		211	121	57.3	1324	28	2.1
4×10^7	30	14	8		214	4	1.9
	31	13	7		210	3	1.4
	32	8	3		213	3	1.4
	33	14	9		206	3	1.5
	34	11	8		Not counted	—	—
	35	10	5		232	3	1.3
	36	Not counted	—		Not counted	—	—
		70	40	57.1	1075	16	1.5
4×10^6	39	2	1		203	2	0.99
	40	6	2		242	1	0.41
	41	5	0		234	5	2.1
	42	0	0		206	5	2.4
	43	2	0		210	5	2.4
	44	0	0		—	—	—
	45	1	0		204	2	0.98
	46	1	1		211	8	3.8
	47	4	2		209	4	1.9
	48	5	1		208	2	0.96
	49	1	1		225	4	1.8
	50	2	1		212	2	0.94
	51	0	0		Not counted	—	—
	52	3	2		234	6	2.6
	53	0	0		Not counted	—	—
	54	4	2		209	3	1.4
	55	0	0		Not counted	—	—
		36	13	36.1	2807	49	1.7

* Approximately 1×10^6 spleen cells were plated on each Petri dish as described in Materials and Methods to ensure the occurrence of only a single central cell in each plaque.

TABLE III—*Concluded*

Dose of sheep red cells injected	Plate No.	Plaque-forming cells			Non-plaque-forming cells		
		Total plaques recovered	Labeled	Per cent labeled	Total cells counted	Labeled	Per cent labeled
4×10^5	62	3	0		220	5	2.3
	63	2	1		229	4	1.7
	64	1	1		212	5	2.4
	65	0	0		217	7	3.2
	66	1	0		232	7	3.0
	67	0	0		218	9	4.1
	68	1	0		215	5	2.3
	69	0	0		207	7	3.4
	70	2	0		209	5	2.4
	71	0	0		231	4	1.7
	72	1	0		227	3	1.3
	73	0	0		216	4	1.9
	74	1	0		216	6	2.8
	75	1	0		219	4	1.8
	76	2	1		218	8	3.7
	77	1	0		243	4	1.6
	78	0	0		207	6	2.9
	79	1	0		224	3	1.3
	80	0	0		234	5	2.1
		17	3	17.6	4194	101	2.5

All the plaque-forming cells as well as non–plaque-forming cells used in the present studies could be classified as lymphocytes or plasma cells, though detailed classification of heavily labeled cells was not possible. Non–plaque-forming granulocytes and macrophage-like cells which were seen rarely in the background were not counted for the purpose of the present study.

Determination of Mean Grain Counts Over Labeled Plaque-Forming and Non–Plaque-Forming Cells.—The same radioautographs from the five separate experiments that had been used to determine proportions of labeled cells were reexamined after storage for several months. To avoid any interobserver error in grain counting, counts were made by a single observer who had not participated in the previous scoring of labeled and unlabeled cells. The absolute numbers of plaque-forming and non-plaque-forming cells recorded for the purpose of grain counting by the single observer differed slightly (cf. Tables II and VI) from those obtained independently by the three observers for the purpose of determining the percentages of labeled plaque-forming and non-plaque-forming cells. Reasons for these differences are as follows: plaques were reevaluated independently by the single observer from coded plates; some plaques were lost as a result of peeling of the agar layer after prolonged storage; larger samples of labeled non-plaque-forming cells had to be collected for the purpose of determining mean grain counts than had been tabulated previously for the purpose of determining the per cent of labeled non-plaque-forming cells.

Determination of Background Grains.—In each experiment, spleen cell suspensions that had never been exposed to tritiated thymidine were plated in thin layers as described above. These

control plates were processed exactly as the experimental plates for radioautography. Some of the control plates were developed as soon as the photographic emulsion had dried (approximately 2 hr at room temperature in the darkroom under Wratten series 1 filters), while other control plates were dried, packed in metal cans with drierite, and subsequently incubated at 4°C for up to 9 days to allow for exposure of the emulsion to any background radiation. Background grain counts were usually low (< 1 grain per cell) in plates that were developed immediately as well as in plates that had been incubated at 4°C for 9 days.

Empty Petri dishes could not be coated with NTB3 emulsion because the emulsion did not adhere permanently to the plastic surface. Peeling of emulsion occurred either when developer or fixer was added or when plates were washed. However, such controls were not considered essential to the present study.

Hemolytic Plaque Assay.—Plaque assays were performed according to the technique of Jerne et al. (6, 7) on aliquots of the same spleen cell suspensions that were processed for radioautographic analyses. In each experiment, samples of cell suspensions were taken before and after 30 min incubation at 37°C to check for loss of plaque-forming cells.

RESULTS

Identification of Labeled Plaque-Forming Cells.—Plaque morphology was maintained in radioautographic preparations as shown in Fig. 4 *a*. Plaque-forming cells could be identified easily in the centers of plaques. The conditions chosen resulted in very heavy labeling of those cells that had incorporated isotope into acid-insoluble intracellular material, whereas unlabeled cells usually had no grains over them at all. Cells could be categorized readily as labeled or unlabeled. In the same microscopic field shown in Fig. 4 *b*, a heavily labeled plaque-forming cell as well as several completely unlabeled non–plaque-forming cells can be distinguished.

Background grain counts were usually very low as determined in control plates. Any cell having fewer than 10 grains was classified as unlabeled; however, in all experiments described in the present study, most labeled cells had 20 or more grains over them.

Proportion of Labeled Plaque-Forming Cells From Spleens of Mice Injected With Different Doses of Sheep Red Cells.—In the great majority of plaques there was only a single central cell. In some plaques, however, there were two contiguous central cells.[2] If both cells were labeled or both cells unlabeled, the plaque could be classified as labeled or not labeled; if, however, one cell was labeled and the other unlabeled, the plaque could not be used in the present study. The latter category comprized only 3% of all plaques observed and will be considered under Discussion.

The results of individual experiments were very reproducible in spite of many variables, some of which are listed in Table I. The proportions of tritiated thymidine-labeled plaque-forming and non–plaque-forming cells obtained from mice inoculated with different doses of sheep red cells are shown in five separate experiments in Table II and Fig. 1. In each experiment, the mice inoculated

[2] Koros, A. M. C., and M. J. Mowery. Manuscript in preparation.

with 4×10^5 sheep red cells yielded the lowest percentage of labeled plaque-forming cells. The percentages of labeled plaque-forming cells were found to increase with increasing doses of antigen injected. This increase was observed up to the dose of 4×10^7 sheep red cells. The spleens of mice injected with either 4×10^7 or 4×10^8 sheep red cells exhibited similar percentages of labeled plaque-forming cells; this is not surprising, for in the dose-response experiments (1–2)[1] it was noted that the spleens of mice injected with either 4×10^7, 4×10^8, or 4×10^9 sheep red cells yielded the same numbers of plaque-forming cells.

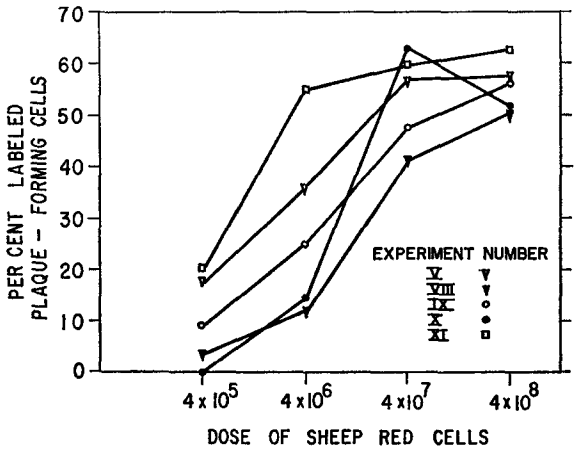

FIG. 1. Per cent labeled plaque-forming cells from spleens of mice injected with different doses of sheep red cells. Each curve represents a separate experiment. The numbers of cells counted per point are shown in Table II.

The percentages of labeled non-plaque-forming cells, on the other hand, did not appear to increase with increasing doses of antigen (Table II). At the three highest antigen doses, 4×10^6, 4×10^7, and 4×10^8, the percentages of labeled plaque-forming cells were always higher than the percentages of labeled non-plaque-forming cells. At the lowest antigen dose tested, the percentage of labeled plaque-forming cells was higher than the percentage of labeled non-plaque-forming cells in two out of five experiments.

The recovery of total plaques as well as recovery of the fraction of labeled plaques from plate to plate within a given experiment was also very reproducible as shown by the results of all the plates examined in Experiment V (Table III).

The total numbers of plaque-forming cells recoverable in the radioautographs of five separate experiments, as well as the fractions of labeled plaque-forming and non-plaque-forming cells, are shown in Table IV. The percentages of labeled plaque-forming cells are significantly higher than the percentages of

labeled non-plaque-forming cells in mice inoculated with high doses as well as in mice inoculated with low doses of sheep red cells.

TABLE IV

*Comparison of Per Cent Labeled Plaque-Forming and Non- Plaque-Forming Cells from Spleens of Mice Injected with Different Doses of Sheep Red Cells**

Approximate dose of sheep red cells injected	Plaque-forming cells			Non-plaque-forming cells		
	Total counted	No. labeled	Per cent labeled	Total counted	No. labeled	Per cent labeled
4×10^8	1148	630	54.9	5,835	241	4.1
4×10^7	370	199	53.8	7,639	293	3.8
4×10^6	256	99	38.6	20,825	704	3.4
4×10^5	83	9	10.8	13,777	691	5.0

* Data compiled from experiments V, VIII, IX, X, XI shown in Table II.

Proportionality Between Dose of Antigen Injected and Percentage of Labeled Plaque-Forming Cells.—The percentage of labeled plaque-forming cells recovered from spleens of mice inoculated with sheep red cells is proportional to the log of the dose of antigen injected up to a dose of 4×10^7 sheep red cells (Fig. 2). There is no significant difference at the 95% confidence level between the per cent of labeled plaque-forming cells at doses of 4×10^7 and 4×10^8 sheep red cells.

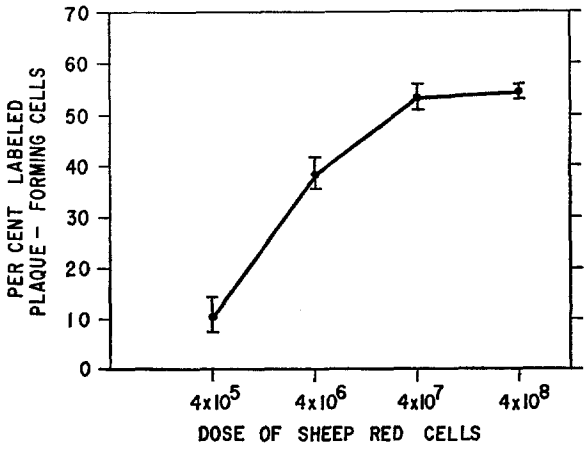

FIG. 2. Effect of antigen dose on recovery of ^{3}H-thymidine-labeled plaque-forming cells. Each point represents the proportion of labeled plaque-forming cells obtained in a total of five experiments shown in Table II. The vertical bars represent ± 1 standard error.

Effect of Incubating Spleen Cell Suspensions 30 min at 37°C on Recovery of Plaque-Forming Cells.—To rule out the possibility that during the incubation period required for isotope incorporation there was selection of a particularly resistant fraction of labeled plaque-forming cells from spleen cell suspensions of mice inoculated with high doses of antigen, the following control was included in each experiment. Samples of spleen cell suspensions taken before and after 30 min incubation at 37°C were assayed on base layers. There were no significant differences between the numbers of plaques recovered per 10^8 spleen cells before and after 30 min incubation in vitro (Tables V A and V B). The numbers of plaques obtained per 10^8 spleen cells in the current experiments were within the range of values obtained earlier (1–2).[1]

Although the proportions of plaque-forming cells were not affected by incubation, the total numbers of spleen cells recoverable after incubation varied from 27 to 98%. The loss in cells was unavoidable and not due to the incubation itself, for similar cell losses were observed in preparations that were not incubated but merely centrifuged and resuspended in Eagle's medium. Cells were probably lost by adherence to the sides of the 15 ml centrifuge tubes.

Mean Grain Counts of Labeled Plaque-Forming and Non-Plaque-Forming Cells From Spleens of Mice Injected With Different Doses of Sheep Red Cells.—The number of grains per labeled plaque-forming cell was counted in the same five experiments which were designed primarily for the purpose of determining the per cent of labeled cells. For this reason, the grain counts over labeled cells were very high and, although there was great variation from cell to cell, many cells had 100 or more grains over them. Grains were counted over all labeled cells in all five separate experiments by a single observer as described under Materials and Methods. Plates were coded to avoid any bias. Each cell was counted three times; the grain counting error was no greater than $\pm 10\%$ for cells having up to 100 grains. The counting error for most cells having more than 100 grains was greater than $\pm 10\%$ due to the superposition of grains; cells scored as having >100 grains therefore had a minimum of 100 grains over them, but could have had several hundred grains.

The mean grain counts of labeled plaque-forming cells from mice inoculated with low doses are similar to the mean grain counts of labeled plaque-forming cells from mice inoculated with high doses of antigen (Table VI).

Labeled non-plaque-forming cells from mice injected with high doses of 4×10^8 or 4×10^7 sheep red cells have grain counts that are similar to those of the labeled plaque-forming cells. However, in those experiments which had many cells with accurately countable grains (Experiments V, VIII, and IX; Table VI), the labeled non-plaque-forming cells from mice injected with low doses of 4×10^6 or 4×10^5 sheep red cells have fewer grains over them than the labeled plaque-forming cells. In Experiments X and XI of Table VI, grain counts over most of the labeled non-plaque-forming cells were >100; therefore,

TABLE V A

*Effect of Incubating Spleen Cell Suspensions 30 min at 37°C on Recovery of Plaque-Forming Cells Plated on Agar Base Layers**

Experiment No.	Dose of sheep red cells injected	Before incubation			After incubation		
		Number of spleen cells plated	Total plaques per plate	Plaques per 10^6 spleen cells	No. of spleen cells plated	Total plaques per plate	Plaques per 10^6 spleen cells
V	4×10^8	1.49×10^6	125	9400	1.21×10^6	225	16700
		1.49×10^6	151		1.21×10^6	196	
		1.49×10^6	142		2.42×10^6	369	
	4×10^7	1.50×10^6	48	3300	1.05×10^6	54	5500
		1.50×10^6	53		1.05×10^6	60	
		1.50×10^6	46		2.10×10^6	116	
	4×10^6	6.60×10^7	183	450	1.92×10^7	100	520
		6.60×10^7	217		1.92×10^7	104	
		4.95×10^6	37		1.01×10^7	51	
	4×10^5	5.24×10^7	144	290	4.16×10^7	74	170
		5.24×10^7	138		4.16×10^7	69	
		2.62×10^7	88		1.56×10^7	27	

* Data obtained from a single experiment (No. V). Data from five separate experiments summarized in Table V B.

TABLE V B

Effect of Incubating Spleen Cell Suspensions 30 min at 37°C on Recovery of Plaque-Forming Cells Plated on Agar Base Layers

Dose of sheep red cells injected	Plaques per 10^8 spleen cells*		t test
	Before incubation	After incubation	
4×10^8	19396 ± 7625	16784 ± 4144	No significant difference at 5% level
4×10^7	3240 ± 1172	4841 ± 2395	" "
4×10^6	274.6 ± 74.4	365.0 ± 118.6	" "
4×10^5	153.2 ± 55.7	149.6 ± 63.1	" "

* Data from experiments V, VIII, IX, X, XI. Mean values ± one standard error.

differences in grain counts among labeled non-plaque-forming cells in those experiments probably would not be detectable.

Effect of Incubating Spleen Cell Suspensions With Tritiated Thymidine for Different Lengths of Time.—The rate of uptake of tritiated thymidine into plaque-forming spleen cells is linear for periods from 15 min to 60 min in vitro

as reflected by the linear increase in the mean grain count over labeled plaque-forming cells (Table VII, Experiment IV, and Fig. 3). The increase may be linear beyond 60 min, but cells were very heavily labeled at 120 min. The error is very great in counting more than 80 grains per cell; thus, the leveling off in grain count may be more apparent than real.

The percentage of labeled plaque-forming cells does not increase significantly

TABLE VI

Mean Grain Counts over Labeled Plaque-Forming and Non-Plaque-Forming Cells Obtained from Spleens of Mice 3 Days after a Single Injection of Sheep Red Cells

Experiment No.	Dose of sheep red cells injected	Plaque-forming cells			Non-Plaque-forming cells		
		Total countable cells	No. labeled	Grains per labeled cell	Total cells counted	No. labeled	Grains per labeled cell
V	4×10^8	233	131	78	1600	35	82
	4×10^7	95	52	81	1600	29	83
	4×10^6	39	13	61	4200	45	53
	4×10^5	16	2	76	4815	114	47
VIII	4×10^8	191	89	83	1200	63	96
	4×10^7	61	25	86	2000	55	88
	4×10^6	21	2	71	1700	64	51
	4×10^5	19	1	>100	2030	88	92
IX	4×10^8	166	88	71	1000	46	89*
	4×10^7	87	45	70	1600	56	92*
	4×10^6	22	4	78	1600	76	98*
	4×10^5	22	2	81	1700	183	69‡
X	4×10^8	199	99	93	1900	129	99*
	4×10^7	44	28	93	2000	126	98*
	4×10^6	40	8	64	4000	172	97*
	4×10^5	10	0	—	4000	161	99*
XI	4×10^8	166	103	92	2000	82	99*
	4×10^7	122	68	92	1700	80	99*
	4×10^6	145	85	91	4100	85	97*
	4×10^5	20	5	86	4300	85	97*

The data recorded above were compiled independently by a single observer from the same five separate experiments shown in Table II and Fig. 1. The data shown in Table II were obtained by three observers. The absolute numbers of plaque-forming and non-plaque-forming cells recorded by the different observers differ for reasons discussed in the text, but the proportions of labeled cells recorded by the single observer are not significantly different from the data compiled by the three observers.

* Most labeled cells counted had > 100 grains per cell.

‡ More than half of the labeled cells had grains in a countable range as described in text.

TABLE VII

Effect of Incubating Spleen Cell Suspensions with 20 μc of Tritiated Thymidine at 37°C for Different Lengths of Time*

Experiment No.	Time incubated with tritiated thymidine	Plaque-forming cells				Non-plaque-forming cells			
		Total counted	No. labeled	Per cent labeled	Mean grain count per labeled cell	Total counted	No. labeled	Per cent labeled	Mean grain count per labeled cell
	min								
IV‡	15	235	108	46	28	1587	63	4.0	Not done
	30	247	129	52	38	1357	46	3.4	"
	60	195	100	51	59	1356	50	3.7	"
	120	187	108	58	87	1376	59	4.3	"
XI‡	30	167	104	62	92	2000	82	4.1	99
	120	230	148	64	94	1900	72	3.8	99
	240	98	58	59	92	1500	49	3.3	>100
	420	20	12	60	98	1750	19	1.1	99

* Spleen cells in each experiment were pooled from five mice 3 days after a single intravenous injection of 4×10^8 sheep red cells; aliquots were incubated for different lengths of time with isotope, washed, and plated as described in Materials and Methods.

‡ Grain counts over labeled cells were done on coded plates by a single observer to avoid error in grain counting.

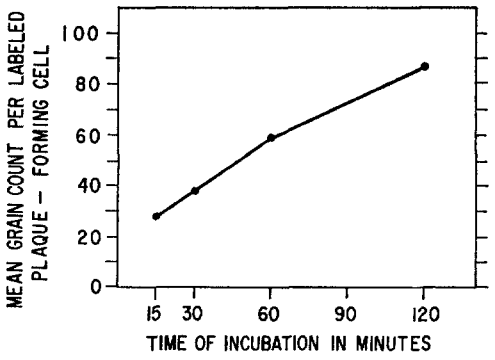

FIG. 3. The incorporation of ^{3}H-thymidine into plaque-forming cells in vitro. Each point represents the mean grain count (shown in Table VII) per labeled plaque-forming cell.

with increasing time of in vitro exposure to isotope for periods from 15 min to 7 hr as shown in Experiments IV and XI in Table VII. Cell suspensions that were kept as long as 7 hr in vitro with isotope had no greater percentage of plaque-forming cells labeled than did suspensions incubated for the standard 30 min at 37°C.

The percentage of labeled non-plaque-forming cells also does not increase with time of in vitro incubation with tritiated thymidine, as shown by the results of Experiments IV and XI in Table VII.

Although spleen cells are not lost as a result of incubation in vitro for periods up to 2 hr, there is appreciable loss of plaque-forming cells beyond 2 hr under our in vitro conditions.

DISCUSSION

The mechanism by which plaque-forming cells appear at an exponential rate in mouse spleens after a single injection of heterologous erythrocytes has been the subject of controversy for several years (references 1–4 and footnote 1; references 6, 9–13).

The experiments described in the present report were designed to answer the following questions: (*a*) Does the exponential increase in plaque-forming cells in vivo in mouse spleen following a single injection of sheep red cells result from cell proliferation? (*b*) Do the increasing exponential rates of appearance of plaque-forming cells in spleens of mice injected with increasing doses of sheep red cells represent proliferation of plaque-forming cells at rates which are dependent on the dose of antigen? (*c*) If high doses of antigen do increase the rate of proliferation of plaque-forming cells, which stage of the cell cycle is traversed more rapidly?

The results presented in this paper indicate that plaque-forming cells proliferate and that the rate of proliferation is dependent on the dose of antigen. The results presented in this paper demonstrate conclusively that 3 days after a single large intravenous dose of 4×10^8 sheep red cells, at least 55% of plaque-forming cells in mouse spleens are capable of incorporating tritiated thymidine into acid-insoluble intracellular material. Such cells presumably have incorporated tritiated thymidine into DNA[3] during the S period of the cell cycle (14, 15). The exponential increase in plaque-forming cells in vivo, therefore, cannot be the result of differentiation alone in the absence of cell division.

The 55% labeled plaque-forming cells obtained from mice inoculated with a high dose of antigen represents a minimum per cent of plaque-forming cells that could synthesize DNA in the mouse for the following reasons: Some spleen cells are injured in the preparation of single cell suspensions (90% of such cells have been shown to be viable by trypan blue exclusion [7]); therefore, no more than 90% could be expected to be labeled under ideal conditions for pulse-labeling. The in vitro conditions described for pulse-labeling are not ideal. If in vitro incubation is prolonged beyond 30 min, the pH of the medium drops

[3] Fresh solutions of tritiated thymidine were used to avoid the incorporation of breakdown products of radioactive thymidine into macromolecules other than DNA as described by other workers (16).

markedly. Some cells settle to the bottom of the incubation tube; some cells may adhere to the sides of the tube. Shaking of tubes during incubation perhaps causes further cell damage; cells are incubated without serum; tritiated thymidine may be depleted rapidly in the incubation mixture (17), and may be completely exhausted after 2 hr in our system. Cells may vary in their pools of DNA precursors; some cells may not incorporate extracellular tritiated thymidine at all (18). On the other hand, in spite of the technical limitations of the in vitro conditions, it is apparent that higher percentages of labeled plaque-forming cells can be obtained from spleens of mice earlier than 3 days after immunization with a single dose of 4×10^8 sheep red cells.[4]

The plaque-forming cell populations appear to be proliferating more rapidly than the background non–plaque-forming cell populations. This is true even in mice injected with low doses of sheep red cells, as evidenced by the fact that the percentages of labeled plaque-forming cells are significantly higher than the percentages of labeled non–plaque-forming cells (Table IV).

The fact that higher percentages of plaque-forming cells are labeled than non–plaque-forming cells in the same radioautographic preparations is a more reliable indication of relative rates of proliferation than is the degree of labeling. Previous attempts (9, 11) to determine rates of proliferation by comparing mean grain counts over two such different populations of cells as immature granulocytes and plaque-forming cells may lead to erroneous conclusions for the following reasons: (*a*) The ratio of nuclear cell volume to total cell volume must be considered in any comparative study of different cell types (14, 15, 17); because the mean path of tritium β particles is only 1 μ, geometry therefore affects the actual grain count over cells. (*b*) Lymphocytes have a significantly lower DNA content than immature granulocytes (18, 19). Mature granulocytes also have significantly lower amounts of DNA than do immature band cells (20). It is possible that plaque-forming cells also have less DNA and therefore would not be expected to incorporate as much labeled DNA precursor as immature granulocytes, even if the rates of proliferation of the two cell populations were identical. (*c*) Pool sizes of DNA precursors may differ in band cells and plaque-forming cells thereby resulting in differential uptake of labeled nucleotides.

The percentage of cells labeled by a brief pulse of tritiated DNA precursor represents the ratio of DNA synthesis time to the generation time of the generative cycle in a population of cells that is dividing asynchronously (15, 21, 22). From the dose response experiments alone, the apparent doubling time of plaque-forming cells is 7 hr in mice injected with high doses of 4×10^7, 4×10^8, or 4×10^9 sheep red cells[1] (1, 3, 12). If the doubling time were really 7 hr, the fact that 55% of plaque-forming cells are labeled would mean that such cells would have an S period of 3.8 hr.

[4] Koros, A. M. C., M. J. Mowery, and J. M. Mazur. Manuscript in preparation.

There is an increase in the mean grain count of labeled plaque-forming cells for at least 2 hr in vitro; therefore, the S period must be at least 2 hr and probably is longer.

Attempts to increase the per cent of labeled plaque-forming cells by increasing the time of in vitro exposure to tritiated thymidine did not result in a significant increase in the per cent of labeled plaque-forming cells for periods from 15 min to 7 hr (Table VII). This is a further indication that the S period in plaque-forming cells from mice injected with high doses of antigen is a relatively long part of the cell cycle.

The relative rates of proliferation of plaque-forming cells are dependent on the dose of injected antigen, although the absolute doubling times of the proliferating cell populations cannot be determined with the data currently available. The percentage of plaque-forming cells taken from spleens of mice injected with different doses of sheep red cells that can be labeled by a brief exposure to tritiated thymidine is proportional to the log of the dose of injected antigen up to a dose of 4×10^7 (Fig. 2). This dose of antigen has been shown to give a maximum rate of increase as well as a maximum number of plaque-forming cells in vivo[1] (1, 2). The rate of plaque-forming cell proliferation as evidenced by the percentage of labeled plaque-forming cells apparently also approaches a maximum at a dose of 4×10^7 sheep red cells. Dutton and Eady (23) reported that the rate of DNA synthesis in spleen cell suspensions from rabbits previously immunized to heterologous serum proteins is dependent on the dose of antigen added in vitro. It was not possible to determine from their data, however, whether the numbers of cells involved in DNA synthesis increased or whether the rate of DNA synthesis increased in those cells already making DNA. The results of the present studies provide evidence that in a primary response, the actual numbers of antibody-forming cells that are capable of DNA synthesis increase with increasing dose of antigen. Whether or not the rate of DNA synthesis per cell in our system also increases with increasing dose of antigen is a more difficult question to answer, but one which has been considered.

Assuming that the concentration of tritiated thymidine in our incubation mixture is in sufficient excess, and that the rate of intracellular incorporation of labeled thymidine is not a limiting factor, then an increased rate of DNA synthesis should be reflected in a higher number of grains per labeled plaque-forming cell. As far as can be determined from the heavily labeled plaque-forming cells (Table VI), all plaque-forming cells are labeled to the same degree, even from mice injected with low doses of antigen. In this system therefore, S appears to be constant, if it is assumed all plaque-forming cells must synthesize the same total amount of DNA. In certain other cell systems the rate of transit through S appears to be constant except at the beginning and end of S (18). To prove conclusively that S is constant for all plaque-forming cells, further

experiments would be required using radioautographs that had plaque-forming cells labeled with grains in a more accurately countable range. However, there is great variation in the degree of labeling even among labeled cells within a single group (18, 24),[5] and therefore slight differences between groups would be difficult to detect.

The existence of a small percentage of double cells[2] that are both labeled in the centers of plaques means that those particular cells must have been in S during the 30 min incubation with isotope and must have completed M during the subsequent steps required for plaque development. G_2 and M together, therefore, cannot be longer than 2 hr. M represents a very brief part of most other cell cycles studied (25, 26).

If S and M are constant, and G_2 is very short, then plaque-forming cells from mice injected with high doses of antigen must pass through G_1 more rapidly than do plaque-forming cells from mice injected with low doses of antigen. In other cell systems studied, the most variable part of the generative cycle is G_1 (18, 27, 28); although in the case of mammary gland cells, hormonal stimulation resulted in a shortening of S (29, 30) as well as G_1 (26).

Direct observation (31) of plaque-forming cells undergoing mitosis in vitro provided evidence that there is great variation in division time even among plaque-forming cells. In one case, a single plaque-forming cell became four cells in 1.5 hr, whereas another plaque-forming cell became four cells in 20 hr; however, it is not known whether cells in that system were taken from animals given a maximal or submaximal stimulus. In addition to the variation observed in division time, there appears to be considerable variation in the process of division of plaque-forming cells, which may be unique to antibody-producing cells. Claflin and Smithies (31) observed not only the emergence of daughter cells of unequal size, but also observed a curious fusion of daughter cells in some plaques. The existence of 3% of plaque-forming cells in our system that had one labeled cell and one unlabeled cell could be accounted for by a process of asymmetric division. This question will be considered further[2] and the possibility of nonspecific chance association of two cells even in dilute single cell suspensions must be examined critically.

Although we have provided evidence that the majority of plaque-forming cells in vivo on day 3 after maximum stimulation are capable of DNA synthesis, the ancestry of these cells is still unresolved. The question of whether or not the postimmunization plaque-forming cells arise from the normal plaque-forming cells which exist in nonimmunized conventional (1, 6, 7, 12, 32, 33) as well as germ-free mice[6] is difficult to approach experimentally. We have only preliminary results that 7% (3/44) of normal plaque-forming cells are labeled by a 30 min in vitro pulse of tritiated thymidine. This value is not significantly

[5] Koros, A. M. C., and M. J. Mowery. Unpublished observations.

[6] Nordin, A. A. Personal communication.

different from the percentage of labeled non–plaque-forming cells found in spleens of normal mice as well as mice injected with high doses of 4×10^8 or 4×10^7 sheep red cells.

It has been suggested that immunocompetent cells are stimulated first to differentiate and then to divide (13, 34). Thus far, however, there has been no direct evidence provided to prove whether or not differentiation precedes division in plaque-forming cells in vivo. Dutton and Mishell (35) showed that initiation of a plaque-forming cell response in vitro requires cell division which begins only after 24 hr. In at least one other cell system, i.e. mouse mammary gland in vitro, cell division is a prerequisite for subsequent cell differentiation (36, 37). In a single experiment, plaque-forming cells were studied from mice as early as 2 days after one injection of 4×10^8 sheep red cells. An even higher percentage of plaque-forming cells appeared to be engaged in DNA synthesis on day 2 than on day 3.[4] 71% (119/168) of day 2 postimmunization plaque-forming cells were labeled by a 30 min pulse of tritiated thymidine compared to 52% (209/402) of day 3 postimmunization plaque-forming cells labeled in the same experiment. The fact that only 55% of day 3 postimmunization plaque-forming cells are labeled compared with the higher percentage of plaque-forming cells labeled on day 2 could be a reflection of the waning proliferative capacity of a population of specific immunocompetent cells. It would be of interest to study plaque-forming cells at still earlier times after antigenic stimulation, but problems of collecting a sufficient number of plaque-forming cells 1 day after immunization are formidable.

The lower mean grain counts over non–plaque-forming cells from mice injected with low doses of sheep red cells compared with the high grain counts over non–plaque-forming cells from mice injected with high doses of sheep red cells (Table VI) suggest that non–plaque-forming cells are stimulated first to synthesize DNA and subsequently to differentiate into antibody-forming cells; however, this point merits further investigation.

SUMMARY

A method has been described for obtaining radioautographs of plaque-forming cells. The method permits radioautographic analyses of small numbers of plaque-forming cells amidst large populations of non–plaque-forming cells. Spleen cells that were pulse-labeled with tritiated thymidine could be categorized readily as labeled or not labeled.

Using this method it was found that (*a*) at least 55% of plaque-forming cells which appear 3 days after a maximal stimulus of 4×10^8 sheep red cells are still capable of DNA synthesis, and must have arisen by cell proliferation; (*b*) the rate of proliferation of plaque-forming cells is proportional to the log of the dose of antigen; (*c*) the S period of plaque-forming cells is at least 2 hr, appears to be constant, and is not influenced by antigen dose. The results sug-

gest that antigen stimulates proliferation of plaque-forming cells by hastening their transit through the G_1 phase of the generative cycle.

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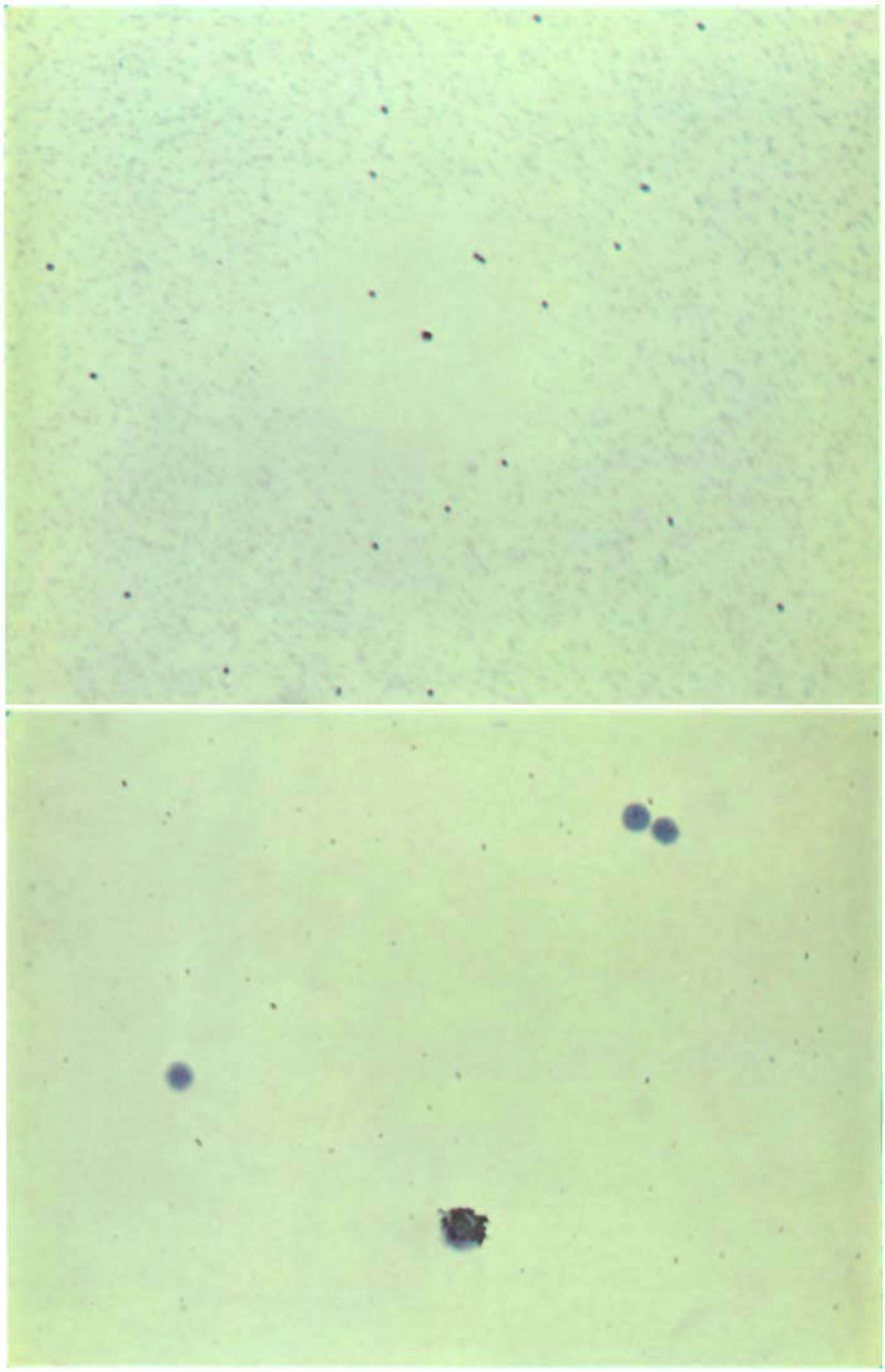
Photomicrographs from Ektachrome type B.

FIG. 4 *a*. Radioautographic preparation of a plaque obtained from a pooled suspension of spleen cells taken from mice 3 days after a single injection of 4×10^8 sheep red cells and labeled with ^{3}H-thymidine. The heavily-labeled plaque-forming cell in the center of the plaque is clearly visible through the approximately 4 μ thick monolayer preparation. Several unlabeled non–plaque-forming spleen cells are visible at the periphery of the plaque as well as throughout the red cell layer. × 140.

FIG. 4 *b*. Enlarged view of the labeled plaque-forming cell and peripheral non-plaque-forming cells shown in Fig. 4 *a*. There are more than 100 grains over the plaque-forming cell nucleus in contrast to the absence of grains over the 3 non–plaque-forming cells at the periphery of the plaque. × 630.



4a

4b