ONSET AND DURATION OF DNA SYNTHESIS IN ANTIBODY-FORMING CELLS AFTER ANTIGEN*

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Plates 3 and 4

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After the injection of antigen into the mammalian host, lymphoid cells divide and increase in number (1, 2). The more rapid appearance of antibody and the greater antibody response after a second antigen injection is thought to be the result of a greater number of cells synthesizing antibody (3). However, a detailed knowledge of the kinetics of these events occurring after antigen injection is not known.

These experiments were designed to determine the onset and duration of DNA synthesis in precursors of antibody-forming cells after a second injection of particulate antigen in the mouse.

Spleen cells were labeled *in vitro* with tritium-labeled thymidine at varying times after exposure to antigen *in vivo*. Antibody-forming cells were identified after transfer to a neutral host by specific fluoroscein stain. DNA synthesized during the earlier *in vitro* period was detected by autoradiography. The results indicate that with the antigen and species used antibody-forming cells began DNA synthesis within 5 hours after the injection of antigen, and continued for at least 24 hours. By 48 hours, all synthesis of DNA in antibody-forming cells has stopped. No DNA synthetic activity before exposure to antigen was detected.

Materials and Methods

Animals and Immunization.—Animals used in these experiments were 8- to 12-week-old B6AF1 mice (Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Maine). They were maintained in air-conditioned animal rooms and fed Purina mouse chow and water *ad libidum*. Bovine gamma globulin (BGG) (fraction II, Pentex, Incorporated, Kankakee, Illinois) adsorbed onto bentonite was used for immunization. The antigen was prepared by the addition of an excess of 2 per cent BGG to a saline suspension of bentonite. The BGG nitrogen of the washed bentonite-BGG complex was determined and primary immunization was accomplished by the intraperitoneal injection of 46 μg BGG nitrogen in 0.1 ml on 3 successive days, 3 to 5 weeks prior to experiment.

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In all experimental groups, the secondary antigenic stimulus of the donor animals was by intravenous injection of 46 μg BGG nitrogen in 0.1 ml bentonite-BGG suspension.

Preparation of Labeled Cells.—Donor animals were anesthetized with ether and killed by exsanguination. Spleen cell preparations were made by gently forcing the spleen through a 60 mesh stainless steel screen. The cell suspension was incubated at 37°C with vigorous shaking for 2 hours in complete N-16 medium (4) supplemented with 25 per cent normal mouse serum (negative for antibody to BGG) and containing 5 μc/ml tritium-labeled thymidine (0.036 mg thymidine/mc tritium, New England Nuclear Corporation, Boston).

At the end of the incubation period, the cells were washed twice with cold buffered physiologic salts and glucose (Saline F., Colorado Serum Company, Denver) and 0.2 to 0.3 ml of cell suspension (35 to 45 × 10^6 nucleated cells) was injected intravenously into the tail vein of 8- to 12-week-old B6AF1 mouse recipients. Recipient mice received 620 roentgens x-irradiation from a Cobalt 60 source 1 day prior to injection of labeled cells. (Our appreciation to the Department of Radiology, University of Colorado Medical Center.) This dose of x-ray inhibits markedly antibody production for at least 2 weeks. Approximately 5600 CPM/ml of tritium-labeled thymidine remained with the cell suspension following two washes. Thus, each mouse received no more than 1700 CPM tritium-labeled thymidine. After 1, 2 or 3 days, (see Results) the recipient mice were sacrificed, their spleens removed and teased apart in 10 per cent normal mouse serum and saline. Brush smear preparations were made on clean slides and the cells were fixed without drying in a 1:1 mixture of ether and 95 per cent ETOH for 15 minutes at room temperature. The slides were then transferred to 95 per cent ethanol for 4 hours at room temperature and air-dried prior to fluorescent staining.

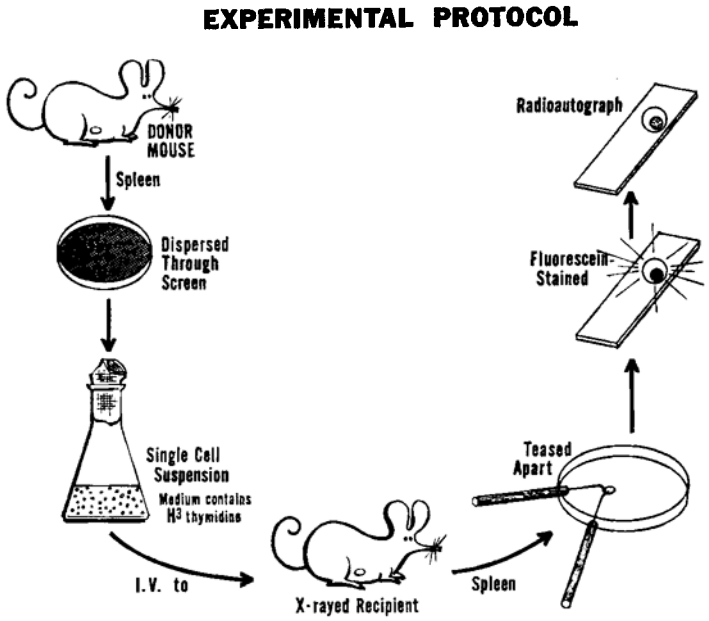
Fluorescent staining.—For specific fluorescent staining, the slides were flooded for 15 minutes with a 2 per cent BGG solution. After washing, the slides were stained for 30 minutes with fluorescein conjugated to antibovine gamma globulin (Colorado Serum Company) and washed again. After air drying, they were examined in a Zeiss fluorescence microscope using an Osram HBO-200 high pressure mercury bulb. Control slides were prepared in an identical manner except the antigen was omitted. Positive cells were photographed and an exact vernier localization was made. At the completion of the slide examination and the identification of antibody-containing cells, the cells were refixed for 1 hour in absolute methanol and prepared for autoradiography. Kodak NTB-3 liquid nuclear track emulsion was melted and diluted 3:1 with 0.1 per cent dupanol. The washed slides were removed from warm triple distilled water and dipped in the emulsion. A Kodak series 2 safelight was used for minimal illumination. The slides were dried for 10 minutes at 40°C and stored in light tight plastic boxes at 4°C for at least 3 weeks.

After storage, the slides were developed in Kodak D-19 for 2 minutes and fixed with hardener edwal quik-fix for an additional 2 minutes. They were then lightly stained with Wright's stain, mounted, and reexamined. With the photograph of the fluorescent cell as a guide, the previously fluorescent cell was localized again without undue difficulty, Figs. 1 *a* and 1 *b*. Cells were scored as labeled if the overlying grain count was 4 grains or more over background.

Four types of fluorescent cells were identified: Fig. 2 *a*, Small cells (nuclear diameter 4 to 6 μ) with a ring of fluorescent cytoplasm; Fig. 2 *b*, Large cells (nuclear diameter 10 to 12 μ) whose nucleus was more centrally located. They had abundant fluorescent cytoplasm; Fig. 2 *c*, Cells with double nuclei, these infrequent cells had two distinct nuclei and a fluorescent cytoplasm; Fig. 2 *d*, Medium sized cells (nuclear diameter 8 to 10 μ) with eccentric nuclei. Frequently, a non-fluorescent nuclear halo was seen. No further attempts toward cell classification were made. Occasional "intermediate" cells were arbitrarily placed in one of these categories.

RESULTS

The use of irradiated isologous recipients eliminates participation by the host in the immune response (5). This was confirmed in the present series of experiments in that recipients receiving antigen alone (without donor cells) formed no detectable hemagglutinating antibody nor was it possible to find antibody-containing cells in the spleens of these animals. However, the examination of the spleens of irradiated mice which had received an intravenous



TEXT-FIG. 1. Antigen was given to the donor mouse intravenously at varying times before removing the spleen (see text).

injection of antigen and cells from a previously immunized donor 2 or 3 days prior to sacrifice revealed frequent cells with antibody.

Recipient mice which had received spleen cells alone (without antigen) from previously immunized donors revealed no detectable antibody-containing cells in their spleens.

The experimental protocol is schematically outlined in Text-fig. 1. To determine the onset of DNA synthesis following antigen injection, antigen was given intravenously to donor mice at varying periods prior to sacrifice. The results are summarized in Table I. Seven per cent of the cells of donor animals given antigen 3 hours before sacrifice (a total of 5 hours prior to injection of

the cells into recipients) were labeled 3 days later. In contrast, 38 per cent of the antibody-forming cells of donor animals given antigen 6 hours prior to sacrifice (a total of 8 hours prior to injection of the cells into irradiated recipients) were labeled 3 days later. The percentage of labeled cells decreased to 19 and 24 per cent in animals killed 12 and 24 hours respectively after injection of antigen. By 48 hours, no antibody-forming cells were labeled. The distribution of cell types identified during these experiments and the number of cells of each type labeled is shown in Table II.

As a test of the technique, spleen cells from mice primarily immunized 5 to 7 weeks prior to transfer were incubated for 2 hours in complete N-16 medium containing 5 μc/ml tritium-labeled thymidine. Antigen was given to the re-

TABLE I

Antibody-Forming Cells Containing Tritium-Labeled Thymidine

Incubation, after antigen to donor	Sacrifice of recipient, after cell transfer	No. of cells with Antibody	No. of cells with tritium-labeled thymidine	Percentage
hrs.	*hrs.*			
3	72	103	8	7.7
6	72	56	21	37.5
12	72	37	7	19.0
24	48	161	38	23.6
48	24	107	0	0.0
48	48	63	2	3.2

TABLE II

Distribution of Antibody-Forming Cell Types

	Small	Large	Double nuclei	Medium
No. of cells with antibody	177	182	32	137
No. of cells with tritium-labeled thymidine	27	31	0	20
Percentage	15.2	17.0	0.0	14.6

TABLE III

Antibody-Forming Cells Containing Tritium-Labeled Thymidine Antigen Given to Recipient at Time of Cell Transfer

Sacrifice of recipient, after cell transfer	No. of cells with antibody	No. of cells with tritium-labeled thymidine	Percentage
hrs.			
48	55	0	0.0
72	54	0	0.0

cipient immediately following the intravenous injection of donor cells and sacrifice was 2 or 3 days later. Recipient spleen cells with antibody were identified and their position carefully noted. Following radioautography, these same cells were relocalized. None were labeled, Table III.

A second series of experiments using C^{14} thymidine to label cells synthesizing DNA and a 3 month exposure of the radioautographs of the labeled cells before examination, confirmed the results in Table I. In addition, no labeled cells were found in groups given antigen 1 or 2 hours before removal of the spleen and C^{14} thymidine incubation.

DISCUSSION

Both histologic (6) and autoradiographic (7) evidences indicate that antigen stimulates the division of lymphoid cells before the appearance of antibody. Baney *et al.* (8), using the double label technique which we have followed, showed that the precursors of antibody-forming cells synthesize DNA.

The present experiments confirm and extend the above observations by describing the kinetics of the induction of DNA synthesis in the precursors of antibody-forming cells. Within 5 hours after the intravenous injection of BGG adsorbed onto bentonite in the primarily immunized mouse, precursors of antibody-forming cells began DNA synthesis.

Gowans (9) showed that an almost pure population of small, non-dividing lymphocytes could transfer the capacity for antibody production to an x-irradiated recipient and induce graft *versus* host reaction in a heterologous recipient. The small lymphocytes localized in the spleen, enlarged, and underwent division. Phytohemagglutinin has also been shown to stimulate mitosis of the small lymphocyte *in vitro* (10).

Evidence indicating the antigen stimulates the synthesis of RNA and protein by a cell already making DNA was obtained by Nossal and coworkers (11). They injected rats with antigen 2 hours after the injection of a large pulse of tritium-labeled thymidine. Nearly 100 per cent of the mature plasma cells obtained from these animals 4 days later was labeled. Nossal reached the guarded conclusion that the plasmablasts arose from large dividing lymphocytes, not small, resting ones. Although they attempted to prevent reutilization of tritium-labeled thymidine by daily injections of large quantities of non-radioactive thymidine, they admitted the possibility that reutilization of labeled thymidine accounts for the high percentage of labeled plasma cells they obtained. However, in their more recent study (12), they found that 100 per cent of the blast cells were labeled as long as 10 days after the injection of tritium-labeled thymidine. This is more readily explained as reutilization than persistence of the original label, since continuous cell division for 10 days would be expected to dilute out to undetectable levels any degree of tritium labeling in the DNA that is compatible with cell life.

In the present experiments, reutilization of tritium-labeled thymidine was avoided by exposing the lymphoid cells to the label only *in vitro* and washing the cells before injection. Thus, the total quantity of label available for reutilization was insignificant. The finding that antibody-forming cells began to incorporate tritium-labeled thymidine within 5 hours after exposure to antigen is consistent with Gowan's contention that antibody-forming cells arise from nondividing cells after the stimulation of DNA synthesis in these cells by antigen.

SUMMARY

Within 5 hours after the intravenous injection of particulate antigen into the primarily immunized mouse, precursors of antibody-forming cells began DNA synthesis as shown by the incorporation of tritium-labeled thymidine. DNA synthesis continued for at least 24 hours and essentially stopped by 48 hours. No DNA synthesis in antibody-forming precursor cells occurred before the injection of antigen.

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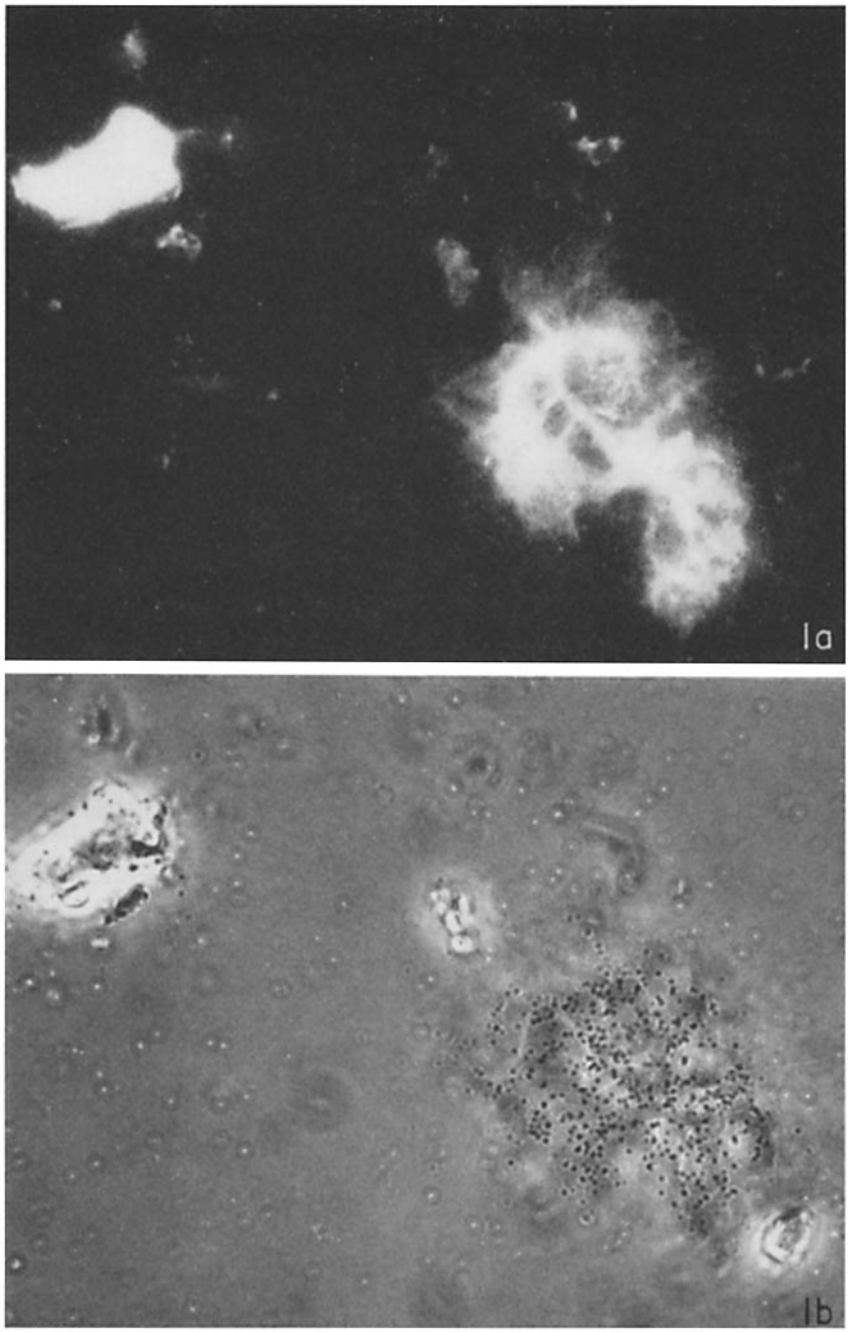
EXPLANATION OF PLATES

PLATE 3

FIG. 1 *a*. Fluorescence micrograph of a small cluster of antibody-forming cells. × 500.

FIG. 1 *b*. Radioautograph of the same cluster showing specific labeling. × 500.



(Cohen and Talmage: DNA synthesis in antibody-forming cells)

PLATE 4

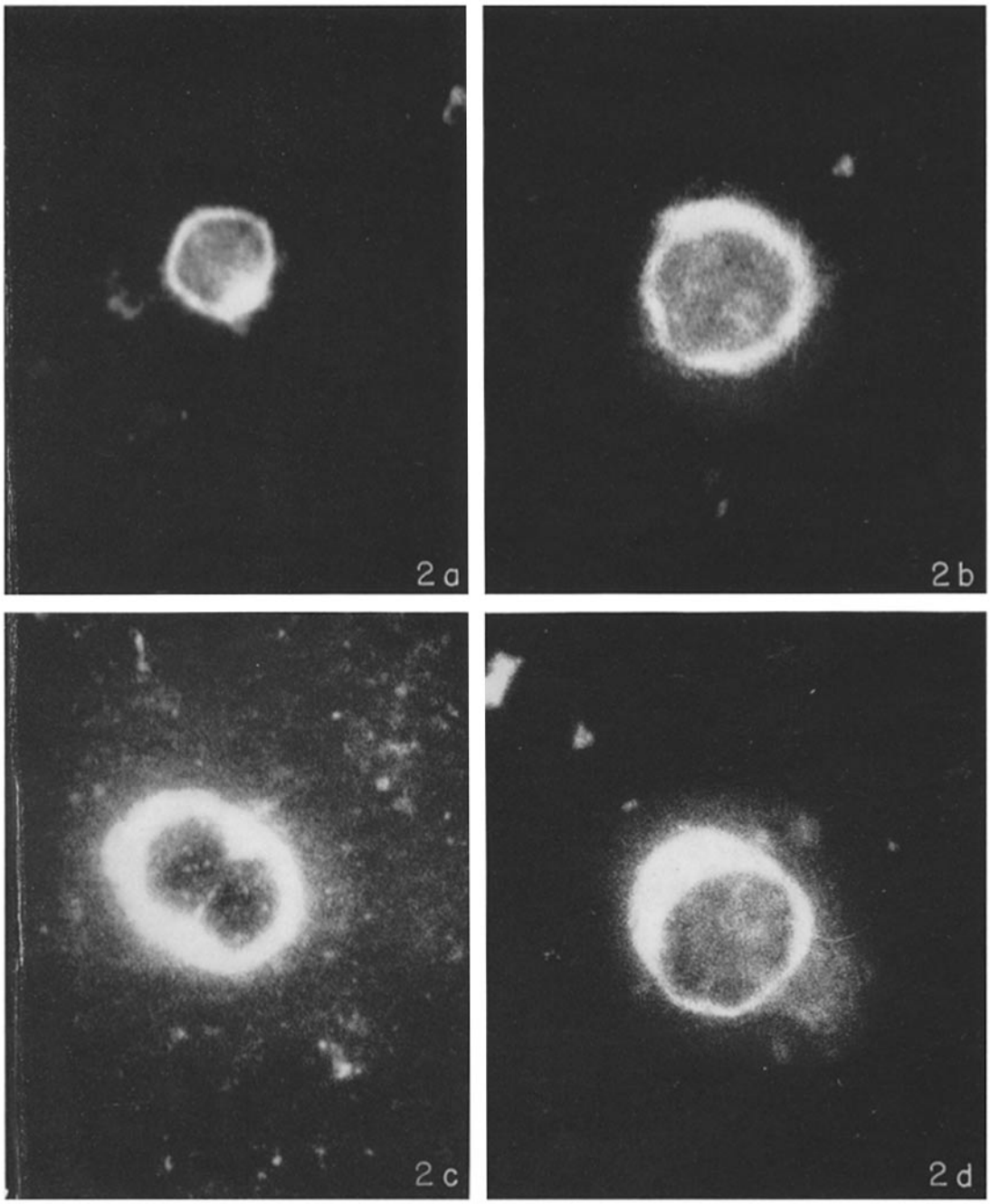
FIGS. 2 *a* to 2 *d*. Fluorescence micrographs of the four cell types ident

FIG. 2 *a*. Small cells. × 600.

FIG. 2 *b*. Large cells. × 600.

FIG. 2 *c*. Double nuclei. × 600.

FIG. 2 *d*. Medium cells. × 600.



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