# STUDIES ON ANTIBODY PRODUCTION*

## VI. The Course, Sensitivity, and Histology of the Secondary Response in Vitro

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Plates 69 and 70

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The preceding paper (1) describes the initial experiments establishing the conditions under which secondary antibody responses may be obtained in tissue culture. The present paper explores the minimal dosage of antigen which is required to obtain a detectable response, and describes the histological appearance of the tissue fragments.

### *Materials and Methods*

Antigens, preparation of lymph nodes and culture media, stimulation *in vitro*, implantation in clotted plasma, incubation, and estimation of antibody titers on fluid withdrawn at 3 day intervals, were similar to those in the accompanying paper (1). In most of these experiments, the fragments were exposed to antigen by incubation before implantation into plasma.

*Histological Methods for Fluorescent Studies.*—To facilitate microscopic examination *in situ*, we occasionally implanted the fragments on a coverslip attached to the wall of the tube by capillarity. In addition, fragments were removed from the tubes at intervals, fixed in 95 per cent ethanol, and paraffin-embedded by the method of Sainte-Marie (2). Fluorescein-conjugated hyperimmune rabbit anti-bovine serum albumin (BSA) and diphtheria (D) antitoxin were purified by absorption with mouse liver powder, or by DEAE cellulose column chromatography by the method of McDevitt *et al.* (3). They were used for the histological localization of the anti-BSA and anti-D (4). Control slides were prepared by omitting the intervening antigen layer. Bouin-Hollande–fixed, paraffin-embedded Dominici-stained sections were used for routine histological examinations (2).

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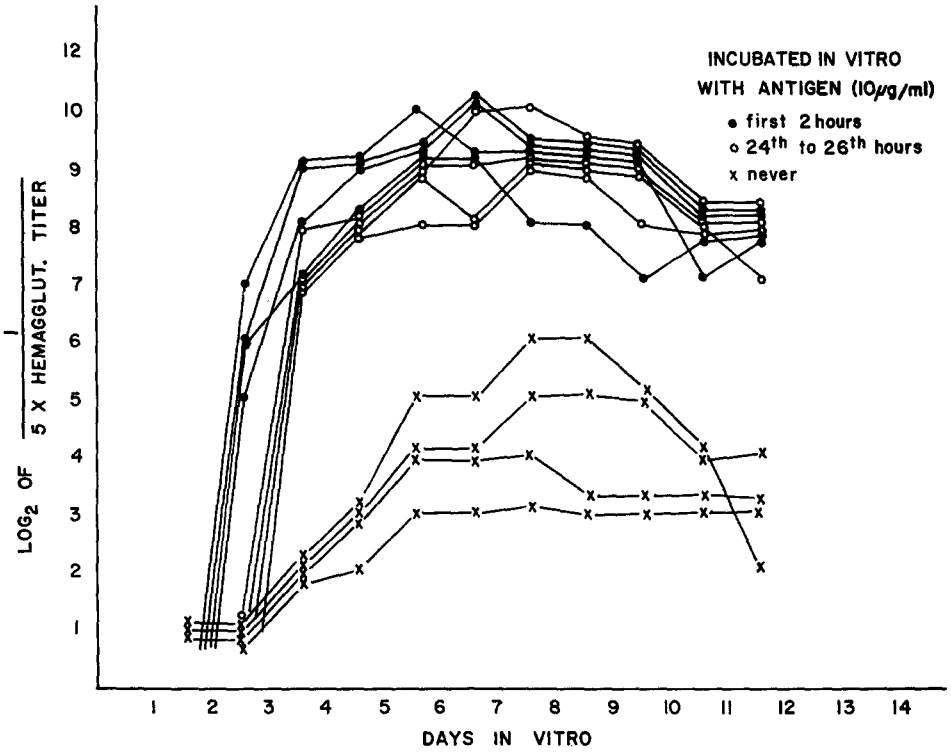
## RESULTS

*Rates of Antibody Production.*—Culture medium in each tube was changed daily and titrated for antibody content. The rise and fall of titer in individual tubes was similar in any one experiment (Text-fig. 1) and also similar from experiment to experiment (Text-fig. 2). After the node fragments were stimu-

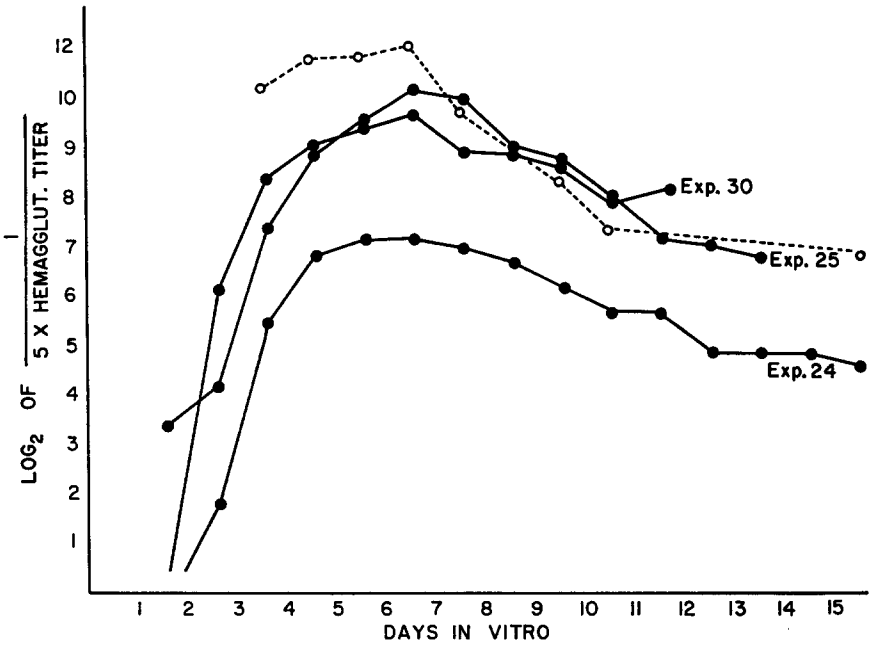



TEXT-FIG. 1. Anti-BSA titers of culture medium changed daily in 12 culture tubes, 4 unstimulated with antigen, 4 stimulated at the time of implantation, and 4 stimulated 1 day later (from Experiment 30).

lated and implanted in the culture tube, they produced little or no detectable antibody for 2 days. On the 3rd or 4th day there was an abrupt rise in the production of antibody, and there was a further rise on the following day. During this 2-day period there was an approximately 30- to 100-fold increase in antibody production. This often doubled again before the peak antibody titer was attained, usually on the 6th or 7th day after antigen stimulation. Antibody titer tended to remain level for another day and then declined slowly; it approximately halved every 2 days. The pattern resembles very closely the time curve of the anamnestic antibody response *in vivo* which

Dixon *et al.* (5) observed in rabbits by studying the incorporation of labeled amino acids into antibody.

In four tubes in which the *in vitro* stimulation was applied 24 hours after implantation, the sharp rise in rate of antibody production took place 1 day later than in the four tubes in which it was applied at the time of implantation (Text-fig. 1). The fragments in four other tubes were not incubated with antigen

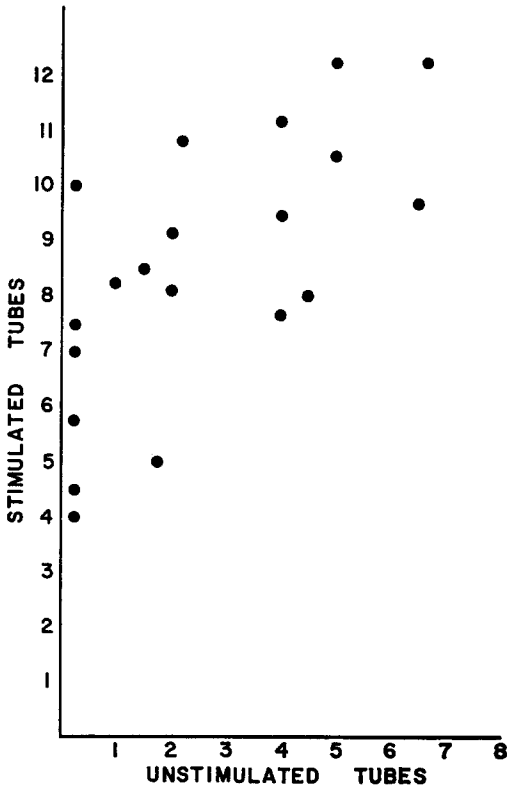



TEXT-FIG. 2. Averages of the $\log_2$ anti-BSA titers of culture medium changed daily in individual tubes stimulated with BSA at the time of implantation in each of 3 separate experiments. The dotted line is a superimposed projection of the values from Dixon *et al.* (5) for incorporation of labeled amino acid into antibody *in vivo* on the corresponding days after stimulation of an anamnestic response.

at any time. Detectable anti-BSA production occurred in these "unstimulated" tubes, but the rate of production rose more slowly, and at its peak averaged only 3 per cent of anti-BSA production in the antigen-stimulated tubes. A similar relationship of anti-BSA production in "stimulated" and "non-stimulated" tubes was noted in a series of twenty experiments (Text-fig. 3). In this figure, for each of twenty experiments the peak titer of the stimulated tubes is plotted against the corresponding peak titer of the "non-stimulated" tubes. Although some antibody was produced by the "unstimulated" tissue in two-thirds of the experiments, stimulation markedly affected the peak titer. In fact, the differences are similar to those seen in the next section where there is a

marked effect of dosage in the lower ranges. In these same experiments, antibody to D was rarely detectable in tubes that had not been stimulated.

*The Effect of Antigen Concentration.*—Table I shows daily anti-BSA production from the 1st to the 6th day of an experiment in which the concentration of the stimulating antigens varied over a wide range. Table II shows the anti-




TEXT-FIG. 3. For each of 20 experiments the average of $\log_2$ anti-BSA titers of culture medium from the stimulated tubes during the period of peak antibody production is plotted against the corresponding average from the unstimulated tubes during the same period.

BSA and anti-diphtheria toxoid titers of culture fluid changed only on the 4th, 7th, and 11th days of an experiment in which the same wide range of stimulating antigen concentration was used. In both experiments an antigen concentration of 0.001 $\mu$g/ml stimulated a detectable but inconstant antibody response. An antigen concentration of 0.01 $\mu$g/ml stimulated a consistent response which averaged about tenfold greater. A further 100-fold increase in antigen to 1.0 $\mu$g/ml resulted in less than a fourfold further increment in average antibody titer.

*Variable Responses in Unstimulated Control Cultures.*—Control cultures, left unstimulated in our experiments, have shown three different patterns of antibody titer. In many of these cultures there was no detectable antibody (*e.g.*, in Table I the anti-diphtheria toxoid titration for control tubes 1 and 2). In other experiments, the control cultures showed a secondary rise in antibody despite the fact that no exogenous antigen had been added. A third pattern of

TABLE I

*Daily Anti-BSA Production in Vitro of Tubes Containing Fragments Stimulated with Varying Concentrations of BSA Just prior to Implantation*

| Concentration of BSA in the "stimulating" medium | Days after antigen stimulation | | | | |
|---|---|---|---|---|---|
| | 2 | 3 | 4 | 5 | 6 |
| *μg/ml* | | | | | |
| 0 (control) | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 |
| 1 | 0 | 10 | 0 | 20 | 40 |
| | 0 | 40 | 40 | 160 | 160 |
| | 0 | 20 | 40 | 80 | 80 |
| 0.01 | 0 | 0 | 0 | 20 | 20 |
| | 0 | 0 | 0 | 20 | 20 |
| | 0 | 0 | 0 | 0 | 10 |
| 0.001 | 0 | 0 | 0 | 20 | 20 |
| | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 |
| 0.0001 | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 | 0 |

response in the control cultures of many experiments was that of a moderate initial titer which fell steadily with successive changes of medium. Such falling titers represent either (*a*) the declining synthesis *in vitro* of antibody from the waning primary response or (*b*) the initial release *in vitro* of antibody from intracellular stores formed *in vivo*.

*Gross and Microscopic Appearance of the Cultures.*—Some of the cultures grown on coverslips in the presence of diphtheria toxoid were examined by histologic and immunofluorescence methods to determine the proportion of cells actively engaged in antibody synthesis, as indicated by their content of antibody.

The changes which took place in such cultures, visible at low magnification of the fragments while still in place, can be briefly summarized as follows: Cell migration appeared to begin very soon after implantation; however, we

TABLE II

*Antibody Production from the 4th to the 7th Day in Tubes Containing Fragments Stimulated in Vitro with Varying Concentrations of Two Antigens Just Prior to Implantation*

| Concentration of each antigen in the "stimulating" medium | Anti-diphtheria toxoid | Anti-BSA |
|---|---|---|
| *μg/ml* | | |
| 0 (control) | 0 | 0 |
| | 0 | 0 |
| | 0 | 10 |
| | 0 | 0 |
| 1.0 | 160 | 640 |
| | 640 | 1200 |
| | 160 | 2500 |
| | 320 | 2500 |
| 0.01 | 640 | 320 |
| | 40 | 80 |
| | 80 | 1200 |
| | 320 | 320 |
| 0.001 | 20 | 20 |
| | 10 | 40 |
| | 40 | 10 |
| | 0 | 40 |
| 0.0001 | 0 | 20 |
| | 0 | 0 |
| | 0 | 0 |
| | 0 | 20 |
| 0.00001 | 0 | 10 |
| | 0 | 20 |
| | 0 | 0 |
| | 0 | 0 |

cannot be certain whether the change in location of cells during the first 2 days was due to active migration or to the slow constant rotation of the tubes. In any event, these dislocated cells consisted of two distinct cell types, both much larger than any cells present in the node at the time of its removal. One was an actively phagocytic cell, large, rounded, occasionally aggregating into giant cells. Some cells of this type had become engorged with erythrocytes and

debris. The other type was long and thin, only as wide as its nucleus. In the nucleus there were two or more nucleoli. Often two or more nuclei appeared to be present in a single elongated cell, especially since the cell boundaries were sometimes difficult to distinguish. The outgrowth of these two cell types continued during the 2nd, 3rd, and 4th week.

In order to detect diphtheria antitoxin in the cells, the original fragments, too thick to stain, were gently removed, and the sheet of cells which had migrated was studied with fluorescent antibody (4). Only a few cells containing antibody were found after 10 days. These resembled mature plasma cells in shape and size. The "dislocated cells" described above did not fluoresce. Control tubes which had not received the antigen showed no fluorescence, nor was toxoid visible in the stimulated tubes.

In later experiments we examined the lymph node fragments themselves (Fig. 1). Sections of these fragments removed at 1 to 2 day intervals from several culture tubes in Experiments 24 and 25 were examined histologically for the presence of anti-BSA. In Experiment 25 ten test slides (stained for antibody) and ten control slides (stained for antigen only) were examined "blindly" by a qualified observer. The evaluations of this observer were: Fragments removed 2 days after antigen stimulation showed no fluorescence; in those removed at 3 days, a few cells with fluorescent cytoplasm were seen; by 4 days, cells with fluorescent cytoplasm were widely and irregularly distributed. Some high-power fields had 20 to 30 such cells with a wide range of fluorescent intensity. Some of these cells had abundant cytoplasm and appeared to be mature plasma cells, but many had relatively large nuclei with a thin rim of fluorescent cytoplasm suggesting a less mature cell. Approximately the same appearance persisted through the 6th day. By the 8th day the observer found the fluorescent cells as numerous as on the 6th and more of them had the appearance of mature plasma cells (Figs. 2 and 3). No fluorescent cells were seen on any of the ten control slides.

Essentially the same sequence had been observed in Experiment 24. A section from the 10th day appeared to have fewer and somewhat fainter fluorescent cells than those of day 6. Fragments were also removed on days 3, 5, and 6 from two tubes which had not been stimulated with antigen. In two of these slides single fluorescent spots were noted, neither of which could clearly be identified as a cell. Control slides showed no fluorescent cells.

## DISCUSSION

In these experiments, daily *in vitro* antibody production rose abruptly on the 3rd and 4th days after antigen addition, and only in tubes containing fragments which had been incubated with the specific antigens *in vitro*. In all experiments, this rise occurred at a fixed time after the fragments had been incubated with a low concentration of antigen and had subsequently been

washed repeatedly. This suggests that continuing appreciable levels of "circulating" extracellular antigen are not required to sustain the developing anamnestic antibody response once it is set in motion by a brief encounter with the stimulating antigen.

The minimum antigen concentration which consistently stimulated a detectable antibody response was 0.01 $\mu$g/ml. The observation that sometimes a response was obtained to as little as 0.001 $\mu$g BSA/ml of medium is a measure of the sensitivity of such "primed" fragments. In fact, the response of such fragments is probably as sensitive a test for antigen as any at present available. These effective doses of antigen in culture parallel the minimum ones long known to be effective *in vivo*.

The charts indicate the production of antibody by fragments in some tubes to which no stimulatory antigen had been added, although it was considerably lower than that in the stimulated tubes. At first, this effect was attributed to traces of antigen on stoppers, test tubes, or in the vessels in which the medium was prepared. However, despite all precautions, antibody formation not infrequently appeared in unstimulated tubes. In view of the very small amounts of antigen necessary to produce a minimum response (0.001 $\mu$g/ml), and of the known persistence of antigen (6), it is our opinion that such responses represent true stimulation by antigen released from sequestered deposits during the cutting procedures necessary to prepare the fragments.

An estimate of the number of antigen molecules which would occupy a single cell volume by simple diffusion at an antigen concentration of 0.001 $\mu$g/ml, assuming a cell diameter of 16 $\mu$, and using 72,000 as the molecular weight of BSA, is 17 molecules per cell. If we further estimate that there are about 10 specific antigenic determinants per molecule, the cell would contain about 200 determinants at the beginning of the synthetic process. According to the data of Leblond and Sainte-Marie (8), and Sainte-Marie and Coons (7), the plasma cell line undergoes about 8 cell divisions, producing therefore 128 cells for each one present at the start (when the antigen was washed off). This means that antigen picked up by simple diffusion could not provide, after division had taken place, more than two antigenic determinants per cell. Histological observations indicate that the concentration of antibody in mature plasma cells is high (9), suggesting that each cell engaged in synthesis does so at a high rate, and must therefore have many active ribosomes.

Haurowitz has long argued that the role of the antigen in antibody production is to act as a template which influences the final folding of the globulin molecule (10). Recently Karush (11) calculated that there are enough disulfide bonds available, taken four at a time, to fix the critical portion of the molecule in about one million different shapes, suggesting that specificity by folding would provide enough different specificities to make this theory feasible. Evidently, another requirement of the template theory is that of a specific con-

centrating mechanism for antigenic fragments, lest the ribosomes have too few determinants to produce the amounts of antibody encountered. Monod (12) has suggested such a mechanism, in analogy with adaptive enzyme formation, to account for the secondary antibody response.

## CONCLUSIONS

The *in vitro* anamnestic antibody response of popliteal lymph node fragments to additions of antigen closely resembles the *in vivo* anamnestic antibody response in its sensitivity to antigen, in the time course of antibody production, and in the sequence of appearance and the morphology of the antibody containing cells.

Most of the cells responsible for antibody synthesis remain in the explant and do not migrate, although a few can be found in the outgrowing sheet of cells.

The smallest concentration of bovine serum albumin which stimulates an anamnestic response *in vitro* is about $1 \times 10^{-9}$ gm/ml.

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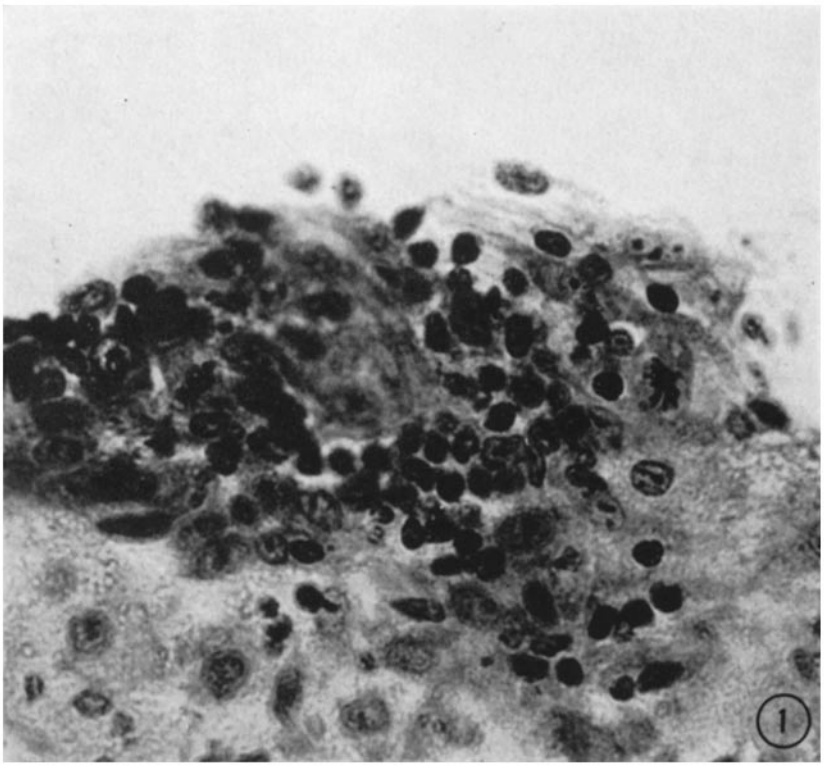
## EXPLANATION OF PLATES

### PLATE 69

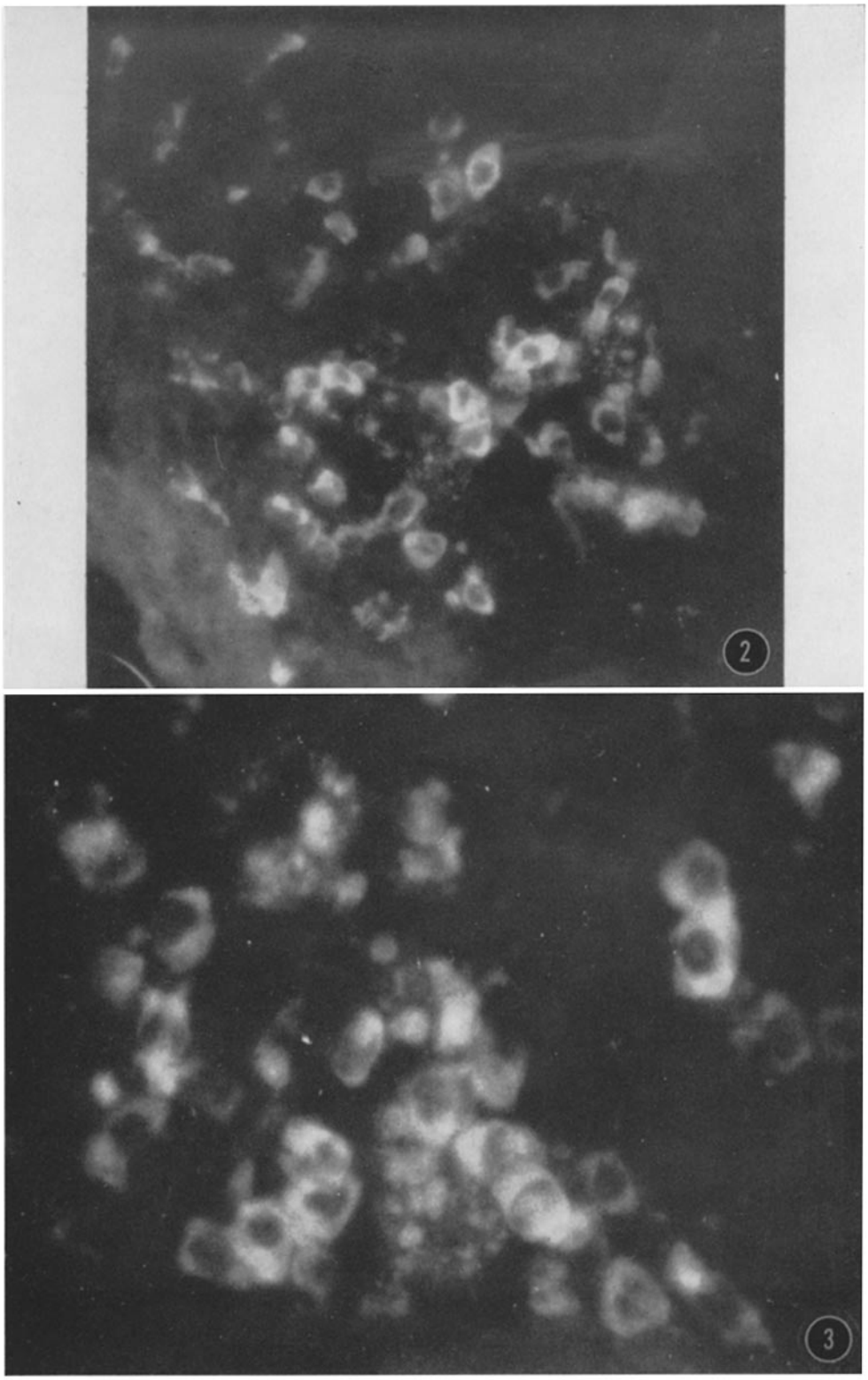
Fig. 1. Section through the edge of a fragment removed from culture on the 2nd day after antigen stimulation and stained with Dominici stain. Two mitotic figures can be distinguished (from Experiment 25). Approximately × 600.




(O'Brien *et al.*: Sensitivity of secondary response *in vitro*)

PLATE 70

Figs. 2 and 3. Sections from fragments removed from culture on the eighth day after antigen stimulation and stained by the immunofluorescent method for detection of antibody (from Experiment 25). Approximately × 600 and × 1200 respectively.




(O'Brien *et al.*: Sensitivity of secondary response *in vitro*)