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Importance of Short-lived Lymphocytes in the Immune Response

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Summary. Lymphocytes are heterogeneous with respect to their life-span. Typical B cells, bearing on their membranes immunoglobulin receptors, easily detectable by immunofluorescence, belong mainly to the long-lived population: this can be observed using combined autoradiography and immunofluorescence. However, when primed mice receive [^{3}H]thymidine before a boosting injection of tobacco mosaic virus (TMV), many plasma cells appearing in the spleen during the secondary response are labelled.

In irradiated recipients repopulated with spleen cells from donors primed with TMV and injected with tritiated thymidine 2 hours before killing, the majority of plasma cells appearing in the spleen after antigen injection were labelled.

If irradiated mice were repopulated simultaneously with spleen cells from donors primed with TMV and injected with [^{3}H]thymidine, and from donors primed with haemocyanin, most of the anti-TMV plasma cells were labelled, while most of the anti-haemocyanin plasma cells were unlabelled. These results allowed us to exclude non-specific reutilization of labelled thymidine as the main reason of our observations. It is concluded that either plasma cells derive from short-lived precursors or they receive material from a labelled cell able to co-operate specifically with plasma cell precursors.

INTRODUCTION

Lymphocytes can be subdivided into two populations, characterized by differentiation antigens (Miller, 1972; Lamelin, Lisowska-Bernstein, Matter, Ryser and Vassali, 1972). Thymus-derived lymphocytes, although they lack easily detectable surface immunoglobulins, can activate or suppress the humoral response to certain antigens. It is commonly believed that during a secondary response, plasma cells are derived from long-lived lymphocytes, bearing immunoglobulin receptors on their membranes (Gowans, 1974; Julius, Masuda and Herzenberg, 1972). Specific antigen could then stimulate cells with the correct receptors to divide rapidly and to synthesize antibody. However, the results of Tannenberg and Malaviya (1968) have shown that the rate of cell proliferation is too low to account for the rapid appearance of antibody-forming cells. Recent data of Marbrook and Haskill (1974) suggest that the immune response to an antigen consists of a continual recruitment of new clones from a pool of precursors.

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Data (Nossal and Mäkelä, 1962) suggesting that plasma cells were derived from a cell population dividing before antigenic stimulation have been presented, but they could be interpreted as being due to thymidine reutilization. Using a virtually non-reutilized precursor, ^{125}I-labelled 5-iodo-2′-deoxyuridine, Miller and Koskimies (1972) have presented data in agreement with those of Nossal and Mäkelä. The aim of this paper was to analyse the relationship between B cells bearing immunoglobulin receptors detectable by immunofluorescence and antibody-producing cells. Our results stress the importance of short-lived lymphocytes in the immune response.

MATERIALS AND METHODS

Antigens

Tobacco mosaic virus (TMV) was isolated following the method of Jeener (1965).

Keyhole limpet haemocyanin (KLH) was purchased from Calbiochem, San Diego, California.

Antibody concentration

This was measured as described by Urbain, Van Acker, De Vos-Cloetens and Urbain-Vansanten (1972).

Cell transfer into irradiated recipients

BALB/c mice (3–9 months old) were irradiated for 3 or 4 hours before receiving donor cells, with an X-ray therapy machine operating under following conditions: 800 R–25 R/minute; 12 mA; 220 kV; additional filtration 0·5 mm Cu and 0·5 mm Al.

Syngeneic donor mice were anaesthetized with chloroform, exsanguinated and their spleens passed through a screen mesh. The cells were suspended in M 199 medium with $NaHCO_3$, containing 25 mg of bactocilline in 100 ml of medium. Cells were washed three times by centrifugation at 1000 rev/minute for 10 minutes at room temperature. The number of viable cells was estimated by the dye exclusion method, using trypan blue. A hundred micrograms of antigen, together with $5–10 \times 10^6$ cells were injected into each irradiated host.

Immunofluorescence

Three fluorescent reagents were used.

(1) Fluorescein isothiocyanate (FITC) purchased from Baltimore Biological Laboratories was coupled to anti-haemocyanin antibodies obtained in rabbits and isolated by precipitation of serum by $(NH_4)_2SO_4$ and chromatography on DEAE–cellulose as described by Cebra and Goldstein (1965).

(2) In the same way, tetramethylrhodamine isothiocyanate (RITC) was coupled to anti-TMV antibodies obtained from rabbits (Urbain-Vansanten, 1970).

(3) FITC coupled to RAM (rabbit antibodies against mouse immunoglobulins) was purchased from Nordic, Antwerp, Belgium.

Mice were killed by exsanguination and their spleens passed through a screen mesh. Cells were suspended in phosphate-buffered saline (PBS) and washed three times by centrifugation at 1000 rev/minute for 10 minutes at 4°. Cytocentrifuge preparations were made on clean glass slides, fixed in 94 per cent ethanol for 20 minutes at room temperature, and air dried. Cells tested for the presence of specific antibodies were covered for 30

minutes with antigen (solution of 2 mg/ml for TMV, and 100 μg/ml for KLH) and then washed twice with PBS. The fluorescent reagent was then allowed to react for 45 minutes, the slides washed twice in PBS, and then rinsed for 5 minutes in 94 per cent ethanol. No cells with cytoplasmic fluorescence were detected if the antigen incubation step was omitted. To detect intracellular immunoglobulins, one incubation of 45 minutes with RAM–FITC was carried out, then slides were washed twice in PBS and rinsed for 5 minutes in 94 per cent ethanol.

Staining for immunoglobulin receptors of live lymphoid cells was performed as described by Loor, Forni and Pernis (1972). Incubation with antigen and fluorescent antibodies were done on cells suspended in PBS. The cells were washed three times after incubation with fluorescent antibodies. Cytocentrifuge preparations of the cells were then made on microscope slides which were fixed for 10 minutes at room temperature in 94 per cent ethanol. A Leitz Orthoplan microscope, equipped with an Opak Fluor vertical illuminator was used to study the stained preparations.

Autoradiography

Autoradiography was carried out on fluorescent stained preparations, as described above. The dry slides were dipped in Ilford K2 emulsion (one-half in distilled water at 42°) and allowed to dry for a few hours on a cold copper plate. Slides were stored for 3 weeks in light-tight boxes at 4°. Autoradiographs were developed for 8 minutes in 0·45 per cent Amidol, fixed in 30 per cent sodium hyposulphite for 6 minutes and rinsed for 1 hour in running water before mounting in buffered glycerine.

Cell labelling

Mice were given a single intravenous injection of 150 μCi of [^{3}H]thymidine (specific activity 5 Ci/mM) in 150 μl of saline. Two milligrams of [^{1}H]thymidine was injected intraperitoneally in 200 μl of saline.

RESULTS

SHORT-LIVED LYMPHOCYTES IN THE SPLEEN OF PRIMED MICE

Lymphocytes may be subdivided into short-lived and long-lived lymphocytes (Everett, Caffrey and Rieke, 1964; Lamelin *et al.*, 1972). After a single intravenous injection of tritiated thymidine (150μ Ci/mouse; 5 Ci/mM) into primed mice, approximately 1 per cent of spleen cells were labelled (Table 1). If primed mice received four thymidine injections, at 8 hourly intervals, approximately 2 per cent of spleen cells were labelled. The exact proportion of short-lived lymphocytes which are labelled cannot be deduced from these data because of the movement of cells in and out the spleen. In addition the analysis of the labelling kinetics of spleen cells with [^{3}H]thymidine is highly complicated (Craddock, Nakai, Fukuta and Vanslager, 1964; Harris, 1973a; Pelc, Harris and Caldwell, 1972). The increased number of labelled cells and the fall in mean grain counts with time after [^{3}H]thymidine injection, suggest that labelled cells were derived from a dividing population. Indeed, 7 days after [^{3}H]thymidine pulsing 20 per cent of cells were labelled, after 8 days 24 per cent, and 10 days after giving thymidine, about 38 per cent of nucleated cells showed more than five silver grains.

Many B lymphocytes possess surface immunoglobulins easily detectable by immunofluorescence. The average proportion of B lymphocytes in the spleen bearing membrane

TABLE 1

PERCENTAGE OF TRITIUM-LABELLED CELLS IN MICE PRIMED WITH TMV, AFTER ONE OR SEVERAL [^{3}H]THYMIDINE INJECTIONS

[^{3}H]Thymidine injections	Number of nucleated scored cells	Number of labelled cells	Percentage of labelled cells
One injection 2 hours before killing	6284	74	1·17
	4842	42	0·86
Four injections at 8 hourly intervals the last injection made 2 hours before killing	4368	64	1·47
	6400	144	2·25
	3560	79	2·22
	5328	93	1·75

immunoglobulins is about 40 per cent. Normal mice and mice primed with TMV (100 μg) 5 months earlier received an intravenous injection of [^{3}H]thymidine, 2 hours before killing. Spleen cells were stained to detect surface immunoglobulins by FITC–anti-immunoglobulin or by TMV and RITC–anti-TMV and processed for autoradiography. The results of one typical experiment out of three are given in Table 2. The results were the same for primed and unprimed mice. Clearly only a small proportion of spleen cells exhibited both membrane immunofluorescence and silver grains. Only 1–2 per cent of labelled cells showed membrane immunoglobulins or TMV receptors, as detected by immunofluorescence. The majority of cells bearing membrane immunoglobulin or binding antigen did not label after a single injection of [^{3}H]thymidine.

NORMAL SECONDARY RESPONSE

Although antigen-binding cells are commonly believed to be the precursors of antibody-producing cells, other studies have suggested that plasma cells are recruited from a dividing cell population. Therefore, mice which had been primed with TMV 1 month before the experiments, were injected with tritiated thymidine 4 hours before a boosting injection of antigen. Unlabelled thymidine was also injected into these mice 2 hours before the boosting injection. At the peak of the secondary response, mice were killed, their spleen removed and processed for intracellular immunofluorescence and autoradiography. In a

TABLE 2

LABELLING OF CELLS BEARING IMMUNOGLOBULINS OR ANTIGEN-SPECIFIC RECEPTORS IN MICE INJECTED WITH [^{3}H]THYMIDINE

Mice*	Incubation of cells	Number of MIg† cells scored	Percentage of labelled cells among MIg cells	Number of labelled cells scored	Percentage of MIg cells among labelled cells
Normal	FITC–anti-IgG	760	0·13	35	2·86
	TMV and anti-TMV antibodies	810	0·25	386	1·04
Primed	FITC–anti-IgG	1186	0·17	199	0·50
	TMV and anti-TMV antibodies	897	0·50	293	1·37

* One injection of [^{3}H]thymidine, given 2 hours before killing.
† MIg = membrane immunoglobulin staining.

typical experiment, on seventy-eight scored plasma cells containing anti-TMV antibodies, 77 per cent were labelled. They were no plasma cells containing anti-TMV antibodies in control and primed mice, not boosted with antigen (see Table 3).

These results are in complete agreement with those of Nossal and Mäkelä (1962) and of Miller and Koskimies (1972). [^{3}H]Thymidine-incorporating cells were heavily labelled (>200 grains/nucleus) 2 hours after administration of this labelled precursor. Plasma cells observed 7, 8 or 10 days after injection of tritiated thymidine, had only fifteen to twenty-five silver grains over their nucleus.

SECONDARY RESPONSE IN IRRADIATED MICE GRAFTED WITH SYNGENEIC PRIMED SPLEEN CELLS

Mice primed with TMV 2 months previous received [^{3}H]thymidine 2 hours before being killed. Their spleen cells were collected, extensively washed, and injected into irradiated syngeneic recipients given an injection of antigen and unlabelled thymidine. The results of three experiments were similar and have been pooled in Table 3. Among 120 plasma cells containing specific anti-TMV antibodies, 101 were labelled. Out of 128 plasma cells stained with FITC–anti-mouse immunoglobulin, 114 were labelled above

TABLE 3

LABELLING OF PLASMA CELLS PRODUCING ANTI-TMV ANTIBODIES DURING A SECONDARY RESPONSE

Experiment	Percentage of labelled plasma cells among total number of scored nucleated cells	Percentage of labelled plasma cells among total number of scored plasma cells
1*	31/3636 (0·85%)	60/78 (76·92%)
Control experiment	0/1384 (<0·07%)	
2†	5/1168 (0·43%)	101/120 (84·17%)
Control experiment	0/2094 (<0·05%)	

* Primed mice injected with [^{3}H]thymidine 2 hours before receiving a boosting injection of antigen.

† Irradiated mice grafted with primed spleen cells and injected with antigen.

background. No plasma cells were found in irradiated recipients given spleen cells from primed mice, but no antigen. The mean concentration of anti-TMV antibodies, in irradiated recipients 1 week after transfer and antigen injection was 220 μg/ml (four recipients were tested). After a second TMV injection, the mean concentration was 550 μg/ml (eleven recipients were tested). Several of these antibodies had a restricted isoelectric pattern, suggesting that a few clones were synthesizing anti-TMV antibodies.

TRANSFER OF TWO SETS OF CELLS TO A SINGLE RECIPIENT

Since the possibility of thymidine reutilization could not be totally excluded by the preceding results, the following experiments were done. In a typical experiment, four donor mice were used. Two mice had been primed 4 weeks before with TMV; two others with KLH. The donors primed with TMV were injected with [^{3}H]thymidine 2 hours before killing; the donors primed with KLH did not receive any radioactive tracer. Irradiated recipients were repopulated simultaneously with cells from TMV-primed donor mice and from KLH-primed mice. The recipients received an injection of both

TABLE 4

PLASMA CELLS IN IRRADIATED HOSTS REPOPULATED WITH TMV-PRIMED LABELLED CELLS AND KLH PRIMED UNLABELLED CELLS

Cells tested for the presence of:	Number of nucleated scored cells	Percentage of fluorescent cells	Number of fluorescent cells scored	Percentage of labelled cells
Anti-TMV antibodies	3636	0·74	132	83·33
Anti-KLH antibodies	4144	0·94	145	17·24
IgG immunoglobulins	3602	4·03	232	52·58

antigens and were injected with cold thymidine. Eight days following transfer, the recipients were killed and their spleen cells processed for combined intracellular immunofluorescence and autoradiography. Results of Table 4 show that 80 per cent of plasma cells containing anti-TMV antibodies were labelled, while less than 20 per cent of plasma cells containing anti-KLH antibodies were labelled. If cells incubated with KLH (or TMV) were stained with fluorescent anti-TMV (or anti-KLH) antibodies, no stained plasma cells could be observed. If spleen cells were stained with FITC–anti-immunoglobulin, about 50 per cent of cells were labelled.

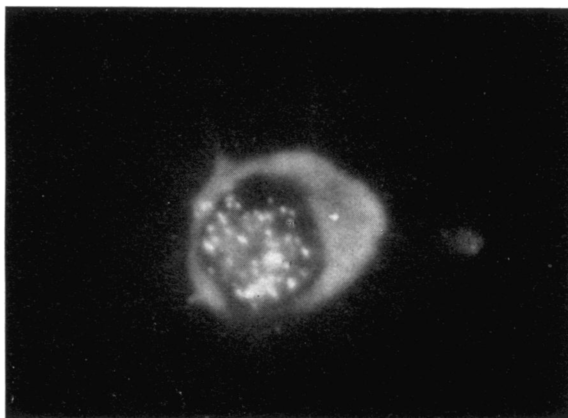

FIG. 1. A labelled plasma cell containing anti-TMV antibodies detected by indirect immunofluorescence and autoradiography. Spleen cells from an irradiated mouse killed 7 days after repopulation with labelled spleen cells from an immunized donor. Recipient received antigen at the time of cell transfer. (Magnification ×3500.)

These transfer experiments of two sets of cells to a single recipient exclude the possibility of thymidine reutilization as the main explanation for our results (Mitchell, McDonald and Nossal, 1963; Rieke, 1962).

A typical plasma cell, labelled with [^{3}H]thymidine and containing anti-TMV antibodies, is shown in Fig. 1.

DISCUSSION

The principal findings reported in this paper can be summarized as follows.

(1) When mice primed several months before, received an intravenous injection of [^{3}H]thymidine before a boosting injection of TMV, most of the resulting plasma cells, containing specific antibody, were labelled (about 80 per cent).

(2) When irradiated recipients were repopulated with spleen cells from donors primed with TMV several months ago and injected with [^{3}H]thymidine 2 hours before killing, the majority of plasma cells appearing in the host after injection of TMV were labelled. When antigen injection was omitted, no plasma cell appeared in the spleen of irradiated mice. Also, when irradiated mice received no cells, no response was observed after antigenic stimulation.

(3) If irradiated mice were repopulated simultaneously with spleen cells from donors primed with TMV and labelled with [^{3}H]thymidine, and from donors primed with KLH, most of the anti-TMV plasma cells were labelled while most of the anti-KLH plasma cells were unlabelled.

(4) If unimmunized mice or primed mice receive an intravenous injection of [^{3}H] thymidine, only a small proportion of B lymphocytes bearing immunoglobulin receptors easily detectable by immunofluorescence were labelled. Therefore, the main conclusions of this work are the following.

(1) The majority of B lymphocytes, bearing easily detectable receptors are not labelled under our experimental conditions. The small proportion of labelled B lymphocytes could be explained by assuming either that this procedure labels some long-lived B lymphocytes in the S phase at the time of thymidine injection, or that a small population of B lymphocytes turns over rapidly. Recent data from this laboratory suggest another interpretation. Typical B lymphocytes with surface immunoglobulins easily detectable by immunofluorescence derive from dividing precursors. These precursors have no membrane immunoglobulins as judged by immunofluorescence. While differentiating and acquiring membrane immunoglobulins, these precursors would lose the capacity to proliferate.

(2) In contrast, the majority of labelled lymphocytes did not bear immunoglobulin receptors detected by immunofluorescence. Of course, this does not prove the complete absence of immunoglobulin receptors since it is known that thymocytes bear receptors which cannot be detected by immunofluorescence (Lamelin *et al.*, 1972).

(3) The most striking finding of these results is that, despite the fact that [^{3}H]thymidine was injected before antigen, plasma cells appearing in response to antigen injection were labelled. Similar results were obtained when the boosting injection was made in the same animal or in irradiated recipients grafted with syngeneic cells from a donor injected with [^{3}H]thymidine. All these results are in agreement with those of Nossal and Mäkelä (1962).

(4) It is known that [^{3}H]thymidine reutilization can occur *in vivo* (Mitchell *et al.*, 1963; Rieke, 1962; Craddock *et al.*, 1964). Some cells having incorporated [^{3}H]thymidine would die and would release some labelled material which could be incorporated into other nuclei preparing for division. This could explain the labelling of a small proportion of plasma cells containing anti-KLH antibodies in the transfer experiments of two sets of cells to a single recipient. However non-specific [^{3}H]thymidine reutilization cannot be the main explanation for our results since 80 per cent of anti-TMV plasma cells are labelled and only 19 per cent of anti-KLH plasma cells are labelled. Furthermore, the labelling of some anti-KLH plasma cells could be explained in an entirely different way. These plasma cells could derive from virgin B cells present in the TMV-primed population. These virgin B cells could be activated by the primed T cells specific for KLH present in the cell population from donors primed with KLH.

Immunological memory has usually been explained by the presence of long-lived

memory cells which are stimulated to proliferate and to differentiate into plasma cells upon re-exposure to antigen. Such a scheme is supported by the studies of Askonas, Williamson and Wright (1970), who were able to propagate a single clone during successive transfers into irradiated mice and by the work of Ellis and Gowans (1973), who demonstrated that long-lived lymphocytes play an important role in immunological memory. Ellis and Gowans (1973) and Julius *et al.* (1972) have suggested that long-lived B lymphocytes are the immediate precursors of plasma cells. We believe that their results clearly showed the importance of long-lived lymphocytes as antigen-sensitive cells but do not exclude the possibility that other lymphocytes could be the precursors of plasma cells. If we suppose that a highly purified population of long-lived lymphocytes is contaminated by 0·1 per cent of short-lived lymphocytes, these lymphocytes, after injection into irradiated hosts, would proliferate rapidly and their frequency will increase rapidly with time. If, as proposed by Julius *et al.* (1972), B lymphocytes bearing high density immunoglobulin receptors are the precursors of antibody-forming cells, we must assume, in order to explain our results, that plasma cells are recruited from the small number of antigen-binding cells which were labelled in our experimental conditions. However, as discussed above, these labelled B lymphocytes most probably represented the end cells of differentiation of B-lymphocyte precursors (C. Bruyns and G. Urbain-Vansanten, manuscript in preparation).

Reutilization of labelled material released by dead or damaged cells cannot be the main explanation for our results, as strongly suggested by the transfer of two sets of cells to a single recipient. However, it cannot be excluded that plasma cells have received some labelled material from a short-lived cell able to co-operate specifically with plasma cell precursors. These cells would be specific for antigen and would co-operate only with plasma cell precursors of the same specificity. Harris (1973b) has shown, using explants of rabbit spleen, that the appearance of antibody-forming cells, as a result of *in vitro* stimulation by erythrocytes, required DNA synthesis but not cell division. He considered it likely that DNA transfer between cells might explain the increased labelling of cells in his study.

Recent studies of Piguet and Vassali (1973) have also stressed the importance of short-lived B cells in immune response. Treatment of primed mice with vinblastine prevents the subsequent stimulation *in vitro* by SRBC or phage T4. On the other hand, it could be that memory cells and antibody-producing cells are different cell lines. Memory cells would belong to a long-lived cell line bearing surface immunoglobulins. Plasma cells would derive from a cell line, dividing constantly and devoid of immunoglobulin receptors detectable by immunofluorescence. Any antibody response would require interactions between these two cell lines.

Current experiments are underway to explore the two possibilities; either plasma cells derive from a short-lived cell line, or they receive labelled material from a specific co-operating cell. The present results, together with those of Nossal and Mäkelä (1962), Miller and Koskimies (1972) and Piguet and Vassali (1973) indicate that the appearance of plasma cells cannot simply be due to the activation of long-lived memory cells in response to antigen.

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