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Control of the Immune Response *in vitro* by Calcium Ions

I. THE ANTAGONISTIC ACTION OF CALCIUM IONS ON CELL PROLIFERATION AND ON CELL DIFFERENTIATION

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Summary. The effects of Ca^{++} on primary and secondary immune responses to SRBC *in vitro* was investigated using the Marbrook technique.

During the primary immune response three periods could be distinguished: first, a Ca^{++}-independent lag period (0–24 hours after antigenic stimulation); second, a period with an absolute requirement for Ca^{++} (24–36 hours after antigenic stimulation), which is related to a proliferative phase of antigenically stimulated cells; and third, a period (later than 48 hours and up to 72 hours after antigenic stimulation), which is inhibited by Ca^{++} and which can be enhanced by removing Ca^{++} from the medium. This third period is related to the differentiation step(s) leading to antibody-forming cells.

During the secondary immune response only a partial inhibition of immune response was observed after removing Ca^{++} from the medium at the time of antigenic stimulation.

Addition of Ca^{++} to EGTA-containing culture medium at any time relative to the initiation of the secondary immune response enhanced the response, but, in contrast to its effects on a primary immune response, never completely restored it. Removal of Ca^{++} later than 6 hours after initiation of the response resulted in a decreased inhibition of the immune response and in an increased switch from 19S to 7S antibody-forming cells. This differentiation step was enhanced by removing Ca^{++} from the medium and was inhibited by Ca^{++} added to the medium.

The results suggest that Ca^{++} controls the mechanisms involved in the antibody formation by an antagonistic action on cell proliferation and cell differentiation.

INTRODUCTION

The requirement of Ca^{++} for lymphocyte stimulation by PHA *in vitro* is well established (Alford, 1970; Kay, 1971; Whitney and Sutherland, 1972). It has been claimed that Ca^{++} is required in the early initiation phase of the reaction (Whitney and Sutherland, 1972). The site and mode of action of Ca^{++} on lymphocyte stimulation, however, is still unknown.

Recently we reported (Diamantstein and Odenwald, 1974) that the primary immune response *in vitro* was inhibited by adding a chelating agent for divalent cations to the

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culture medium at the time of initiation of the immune response. Since simultaneous addition of Ca^{++} restored the immune response, we claimed that Ca^{++} is required for initiation of the immune response. However, an unexpected enhancement of the immune response was detected when Ca^{++} was removed from the culture medium later than 60 hours after antigenic stimulation. The results indicated that Ca^{++} may regulate antibody formation *in vitro*.

The central question of this report was to determine the Ca^{++}-sensitive period(s) during the primary and secondary immune response *in vitro* and if possible to give an interpretation of the presumed regulatory function of Ca^{++} on the immune response.

MATERIALS AND METHODS

Culture medium

For preparation of spleen cell suspensions as well as for cell cultures, a medium consisting of RPMI 1640 medium (Biocult) supplemented with 10 per cent foetal calf serum (Biocult) and L-glutamine (2 mM/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml) was used.

Spleen cell suspensions

Spleen cell suspensions for primary or secondary immune responses *in vitro* were prepared from pooled spleens derived from non-immunized or sheep red blood cell (SRBC) primed female BALB/c mice, 8–10 weeks old.

For priming, the mice were injected with 2×10^8 SRBC per mouse i.p. 14 days before being killed. The spleens were carefully removed, washed three times with saline and teased with forceps in ice-cold culture medium (5 ml/spleen). Single cell suspensions were obtained by separation of teased spleen cells from tissue fragments by filtration through a 100 mesh steel sieve. After centrifugation at 200 ***g*** the cells were resuspended in culture medium (2×10^7/ml).

Cell cultures for anti-SRBC response in vitro

2×10^7 spleen cells were cultured in 1 ml of culture medium with or without 2×10^7 SRBC (three times washed) in Marbrook inserts and placed in an external chamber containing 40 ml of culture medium.

The cultures were incubated in a humidified atmosphere of a CO_2:air ratio of 5:95 at 37° for 2–4 days (usually 4 days). Ethyleneglycol-*bis*-(2 aminoethylether)-*N*,*N*′-tetra-acetic acid (EGTA) and/or $CaCl_2$ were added to the medium in the external chamber at various concentrations (usually 0·7 mM) and at various times relative to the addition of SRBC to the spleen cells.

Assay for plaque-forming cells (PFC)

The cells were harvested from the Marbrook inserts, washed with culture medium, and resuspended in 1 ml of phosphate-buffered MEM medium (Diamantstein, Wagner, Beyse, Odenwald and Schulz, 1971). For detection of direct (19S) plaque-forming cells (PFC) 0·5 ml of spleen cell suspension (or spleen cell suspension diluted 1:10 with phosphate-buffered MEM) was mixed with an equal volume of SRBC (1×10^9/ml, three times washed with saline) and with 0·1 ml of guinea-pig complement (Behringwerke, Marburg). For detection of indirect PFC 0·05 ml rabbit anti-IgG serum (Behringwerke,

Marburg), diluted 1:10 with MEM was added to the mixture containing spleen cells SRBC and complement. A hundred microlitres of the suspension mixture were rapidly placed into the Cunningham chambers (Cunningham and Szenberg, 1968) and incubated for 1 hour at 37°. Controls were incubated without complement or with normal rabbit serum instead of antiserum. PFC were assayed in each individual culture in triplicate. The number of PFC per culture was calculated by multiplication of the arithmetic means of triplicate determinations (standard deviation less than 6 per cent) with the dilution factors.

RESULTS

THE EFFECT OF EGTA CONCENTRATION ON THE PRIMARY IMMUNE RESPONSE TO SRBC *in vitro*

The exogenous Ca^{++} requirement for a primary immune response *in vitro* was determined by variation of the Ca^{++} concentration in the culture medium using EGTA, a

TABLE 1
EFFECT OF EGTA CONCENTRATION ON THE PRIMARY IMMUNE RESPONSE

EGTA concentration (mM)*	SRBC	PFC/culture†
0	−	122 ± 32
0	+	1345 ± 210
0·14	+	1290 ± 180
0·28	+	1411 ± 246
0·42	+	1508 ± 382
0·56	+	1390 ± 270
0·70	+	136 ± 44

* One millilitre of medium containing 2×10^7 spleen cells and 2×10^7 SRBC was placed in Marbrook inserts and cultured with 40 ml of medium containing various amounts EGTA.

† Direct 19S PFC were assayed in 4-day cultures. PFC values represent means of PFC detected separately in three individual cultures ± standard deviation.

chelator which has an affinity for Ca^{++} 10^5 times greater than its affinity for Mg^{++} (Caldwell, 1970). In the culture medium used a concentration of Ca^{++} ions ranging between 0·62 and 0·66 mM was detected (Diamantstein and Odenwald, 1974). The immune response was measured after a 4-day culture period. Concentrations of EGTA ranging from 0·14 to 0·56 mM had no effect on the immune response. However, EGTA at a concentration of 0·7 mM completely inhibited the response (Table 1).

The results summarized in Table 2 demonstrate that the inhibitory effect of EGTA was due to chelation of the free Ca^{++} ions, since simultaneous addition of Ca^{++} ions and EGTA completely restored the immune response. The Ca^{++} requirement for the immune response seems to be specific, since other divalent cations tested, e.g. Mg^{++} and Zn^{++}, were ineffective.

TABLE 2

ABOLITION OF THE INHIBITORY EFFECT OF EGTA BY Ca^{++}*

SRBC	EGTA	$CaCl_2$	$MgCl_2$	$ZnCl_2$	PFC/culture†
−	−	−	−	−	132±42
+	−	−	−	−	1120±230
+	+	−	−	−	152±48
+	+	+	−	−	1070±260
+	+	−	+	−	114±24
+	+	−	−	+	122±36

* Spleen cells mixed with SRBC were cultured for 4 days as described in Table 1 with or without 0·7 mM EGTA. $CaCl_2$, $MgCl_2$ and $ZnCl_2$ at a concentration of 0·7 mM were added to the EGTA-containing medium prior to the start of the culture period.

† Direct 19S PFC were assayed in 4-day cultures. PFC values represent means of PFC detected separately in three individual cultures ± standard deviation.

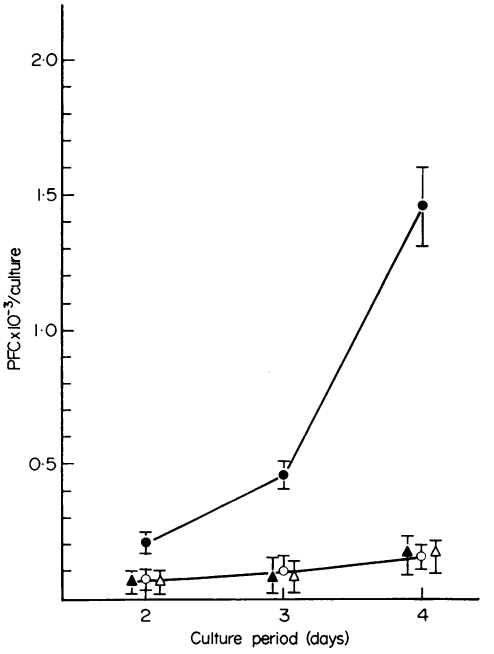

FIG. 1. The effect of EGTA on the kinetics of the primary immune response. 2×10^7 spleen cells with or without 2×10^7 SRBC suspended in 1 ml of medium were placed in Marbrook inserts and were cultured in normal culture medium (without EGTA) or in medium containing EGTA for various times. EGTA at a concentration of 0·7 mM was added to the medium prior to the beginning of the culture period. PFC values represent the mean of direct 19S PFC detected separately in three individual cultures. The standard deviations are shown by the vertical bars. (○) Spleen cells cultured without SRBC in the absence of EGTA. (●) Spleen cells cultured with SRBC in the absence of EGTA. (△) Spleen cells cultured without SRBC in medium containing EGTA. (▲) Spleen cells cultured with SRBC in medium containing EGTA.

Kinetic studies (Fig. 1) demonstrate that the addition of EGTA (0·7 mM) to the medium at the beginning of the culture period completely inhibits the immune response at any time tested, but has no effect on the number of background PFC.

THE TWO Ca^{++}-DEPENDENT PERIODS FOR THE PRIMARY IMMUNE RESPONSE

In order to determine the critical time(s) when Ca^{++} has to be present in the medium in order to permit an immune response two kinds of experiments were performed. (1) EGTA (0·7 mM) was added to the culture medium at various times relative to the start of the immune response by antigen, and (2) to the culture medium containing EGTA (0·7

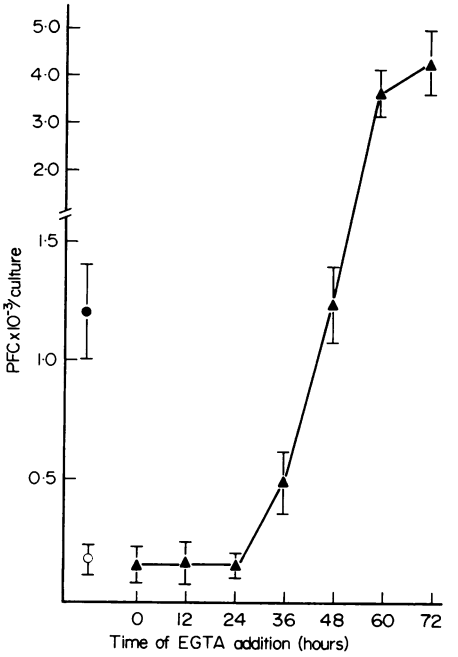

FIG. 2. Effect of adding EGTA at different times relative to SRBC on the primary immune response. 2×10^7 spleen cells were cultured with 2×10^7 SRBC in Marbrook inserts. EGTA at a concentration of 0·7 mM was added to the medium at the indicated times with zero time being the time when SRBC were added to the spleen cells. PFC responses were determined after 4 days of culture. Each value represents the arithmetic mean of direct 19S PFC detected separately in nine individual cultures. The standard errors are shown by the vertical bars. (○) Spleen cells cultured without SRBC in normal medium (without EGTA). (●) Spleen cells cultured with SRBC in normal medium (without EGTA). (▲) Spleen cells cultured with SRBC in medium containing EGTA.

mM), Ca^{++} at a concentration of 0·7 mM was added at various times relative to the start of the immune response. Addition of EGTA up to 24 hours after the start of the cultures completely inhibited the immune response. EGTA added to the medium after 36 hours only partially inhibited the response. Cultures treated with EGTA after 48 hours had unaltered immune responses. However, addition of EGTA after 60–72 hours significantly increased the immune response (Fig. 2).

Addition of Ca^{++} up to 24 hours after antigenic stimulation abolished the inhibitory effect of EGTA. Ca^{++} added to the medium at 36 hours only partially restored the immune

response. Addition of Ca^{++} at any time after 36 hours was ineffective (Fig. 3). Since spleen cells cultured for the last 48 hours in medium containing EGTA gave a normal response, it seems unlikely that the inability of Ca^{++} to restore the response when given 36 hours after EGTA treatment is due to toxic effects of EGTA on the spleen cells. Comparing the results summarized in Figs 2 and 3, it is evident that during the primary immune response two Ca^{++}-dependent periods exist. Since EGTA inhibited the immune response when added up to 24 hours after antigenic stimulation, and because addition of Ca^{++} up to 24 hours to EGTA-containing medium restores the immune response, the first Ca^{++}-

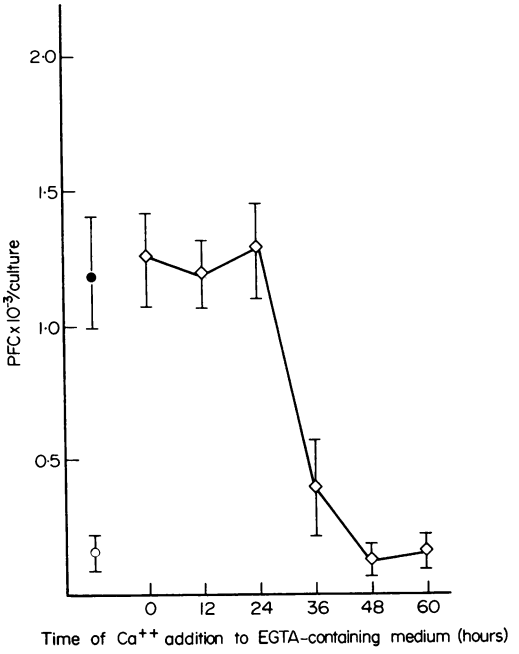

FIG. 3. Effect of adding Ca^{++} at different times relative to SRBC to EGTA-containing medium on the primary immune response. 2×10^7 spleen cells were cultured with 2×10^7 SRBC in Marbrook inserts in EGTA (0·7 mM) containing medium. Ca^{++} at a concentration of 0·7 mM was added to the medium at the times indicated, zero time being the time when SRBC was added to the spleen cells. PFC responses were determined after 4 days of culture period. Each value represents the mean of direct 19S PFC detected separately in nine individual cultures. The standard errors are shown by vertical bars. (○) Spleen cells cultures without SRBC in normal medium (without EGTA). (●) Spleen cells cultured with SRBC in normal medium (without EGTA). (◇) Spleen cells cultured with SRBC in EGTA-containing medium in the presence of Ca^{++}.

dependent step must take place later than 24 hours but less than approximately 36 hours after antigenic stimulation. During this period Ca^{++} has to be present in the medium to permit the immune response. A second Ca^{++}-dependent step occurs after 48 hours of culture period, apparently 60–72 hours after antigenic stimulation.

This step is inhibited by Ca^{++}, since removal of free Ca^{++} by EGTA during this time enhanced the immune response. The prerequisite for this second step is, however, the presence of Ca^{++} during the first Ca^{++}-dependent step.

EFFECT OF EGTA IN SECONDARY IMMUNE RESPONSE

Spleen cells derived from mice primed with 2×10^8 SRBC were cultured with and without SRBC in normal culture medium (without EGTA) or in medium containing EGTA at a concentration of 0·7 mM. The number of direct (19S) and indirect (7S) PFC were detected at various times (2–4 days) after the start of the culture period. The secondary immune response was inhibited by EGTA at any time tested. However, in contrast to the primary response the secondary immune response was only partially inhibited by EGTA (Fig. 4).

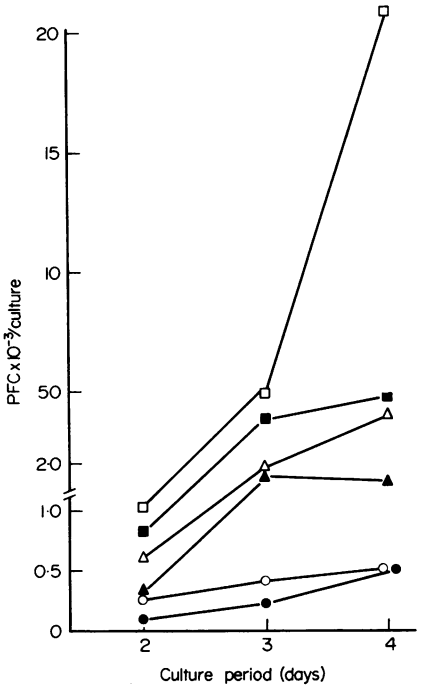

FIG. 4. The effect of EGTA on the kinetics of the secondary immune response. 2×10^7 spleen cells derived from SRBC-primed mice and 2×10^7 SRBC suspended in 1 ml of culture medium were placed in Marbrook inserts and were cultured in normal culture medium (without EGTA) or in medium containing EGTA for various times. EGTA at a concentration of 0·7 mM was added to the medium prior to the start of the culture period. Direct 19S PFC (open symbols) and indirect 7S PFC (solid symbols) were detected each day in three individual cultures. Each value represents the arithmetic mean of PFC detected separately in three individual cultures. Standard errors were less than 20 per cent. (○) and (●) Spleen cells cultured in normal medium without SRBC. (□) and (■) Spleen cells cultured with SRBC in normal medium. (△) and (▲) Spleen cells cultured with SRBC in medium containing EGTA.

THE Ca^{++}-DEPENDENT PERIODS FOR THE SECONDARY IMMUNE RESPONSE *in vitro*

In order to investigate whether critical Ca^{++}-dependent periods may exist in the secondary immune response, as described for the primary immune response, SRBC-primed spleen cells were cultured with or without SRBC in Marbrook inserts with normal culture medium (without EGTA). EGTA (0·7 mM) was added to the medium at various times relative to the antigenic stimulation. In the second type of experiment the Marbrook inserts containing the spleen cells with or without antigen were cultured in medium containing EGTA

(0·7 mM), and Ca^{++} (0·7 mM) was added to the medium at various times after antigenic stimulation. The highest degree of inhibition of the immune response was obtained when EGTA was added between 0 and 6 hours after the start of culture period. Addition of EGTA at progressively later times (Fig. 5) resulted in a decreasing degree of inhibition

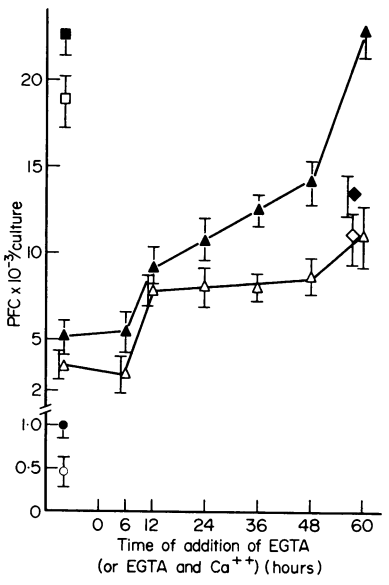

FIG. 5

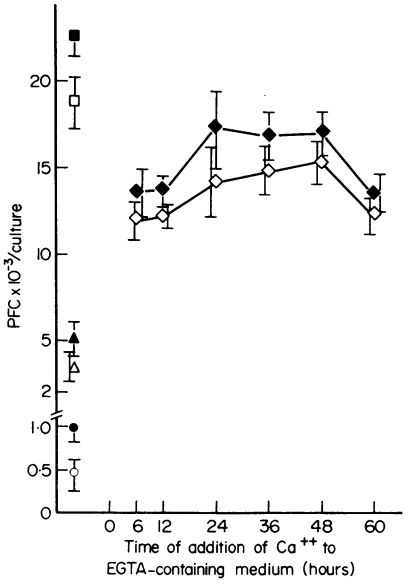

FIG. 6

FIG. 5. Effect of adding EGTA at different times relative to SRBC on the secondary immune response. 2×10^7 spleen cells derived from SRBC-primed mice were cultured with 2×10^7 SRBC in Marbrook inserts. EGTA at a concentration of 0·7 mM was added to the medium at the indicated times with zero time being the time when SRBC were added to the spleen cells. In some cultures 0·7 mM Ca^{++} was added simultaneously with EGTA to the medium at 60 hours of the culture period. Direct 19S PFC (open symbols) and the sum of direct and indirect 19S + 7S PFC (solid symbols) were determined after a 4-day culture period. Each value represents the arithmetic mean of PFC detected separately in nine individual cultures, except of cultures treated simultaneously with Ca^{++} and EGTA. In this case PFC values represent the mean of PFC detected in three individual cultures. The standard errors are shown by the vertical bars. (○) and (●) Spleen cells cultured without SRBC in normal medium (without EGTA). (□) and (■) Spleen cells cultured with SRBC in normal medium (without EGTA). (△) and (▲) Spleen cells cultures with SRBC in medium containing EGTA. (◇) and (◆) Spleen cells cultures with SRBC, Ca^{++} and EGTA added simultaneously to the medium.

FIG. 6. Effect of adding Ca^{++} at different times relative to SRBC to EGTA-containing medium on the secondary immune response. 2×10^7 spleen cells derived from SRBC primed mice were cultured with 2×10^7 SRBC in Marbrook inserts in EGTA (0·7 mM) containing medium on the outside. Ca^{++} at a concentration of 0·7 mM was added to the medium at the times indicated, zero time being the time when SRBC were added to the spleen cells. Direct 19S PFC (open symbols) and the sum of direct and indirect 19S + 7S PFC (solid symbols) were determined after a 4-day culture period. Each value represents the mean of PFC detected separately in nine individual cultures. The standard errors are shown by vertical bars. (○) and (●) Spleen cells cultured without SRBC in normal medium (without EGTA). (□) and (■) Spleen cells cultured with SRBC in normal medium (without EGTA). (◇) and (◆) Spleen cells cultured with SRBC in EGTA-containing medium in the presence of Ca^{++}. (△) and (▲) Spleen cells cultured with SRBC in EGTA-containing medium

and an increasing switch from 19S to 7S antibody-forming cells. Addition of Ca^{++} to EGTA-containing medium from 6 to 60 hours after antigenic stimulation enhanced the response but failed to restore it completely. Comparing the results summarized in Figs 5 and 6 it can be seen that the enhanced switch from 19S to 7S PFC observed in EGTA-treated cultures is inhibited by Ca^{++}.

DISCUSSION

It has been reported that Ca^{++} is required for the mitogenic action of PHA on human peripheral blood lymphocytes, but only during a period of approximately 12 hours after the addition of PHA to the lymphocytes (Whitney and Sutherland, 1972). Both the site of action and the mechanism(s) involved in this effect are still unknown.

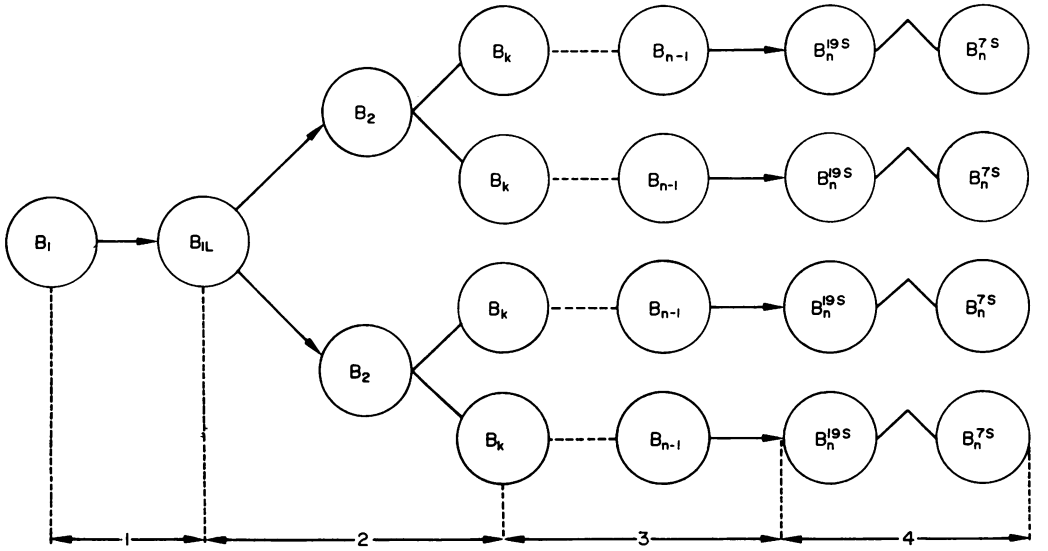

FIG. 7. Regulation of the immune response by Ca^{++} showing the Ca^{++}-dependent and the Ca^{++}-independent periods. (1) Period = lag period, Ca^{++}-independent. (2) Period = cell proliferation, absolute requirement for Ca^{++}. (3) Period = cell differentiation, inhibited by Ca^{++}. (4) Period = cell differentiation, switching from 19S to 7S antibody-forming cells and/or differentiation of 7S precursors to 7S PFC, inhibited by Ca^{++}. B1 = Specific receptor-bearing antibody-forming precursor cell. B_{1L} = The same cell after the lag period. B_2–B_K–B_{n-1} = Cells derived from B_{1L} by clonal cell proliferation. B_n^{19S} = 19S antibody-forming cell derived from B_{n-1} or B_K by differentiation. B_n^{7S} = 7S antibody-forming cell derived from B_n^{19S} by switching or from 7S precursors by differentiation. For detailed explanations see Discussion section.

Recently we reported that EGTA, a chelating agent, highly specific for Ca^{++}, inhibits the immune response to SRBC *in vitro* when it is given to the cultures simultaneously or up to 36 hours after the antigenic stimulation. However, EGTA enhanced the immune response when added to the cultures later than 48 hours after initiation of the immune response. Whitney and Sutherland (1972) claimed that in their system Ca^{++} is involved in processes taking place during the lag period preceding cell proliferation and transformation. As demonstrated in this report for the primary immune response *in vitro* two Ca^{++}-dependent periods can be distinguished. The first Ca^{++}-dependent step correlates with the period of SRBC-induced cell proliferation reported by Dutton and Mishell (1967). However, in contrast to the mitogenic stimulation reported by Whitney and Sutherland (1972), in our system the lag period preceding cell proliferation does not require Ca^{++}. The second Ca^{++}-dependent step takes place 48 hours after the antigenic stimulation. This step (or steps), presumably one of differentiation, is inhibited by Ca^{++}.

Since in the antigen-stimulated cultures Ca^{++} is not required during the lag period, it

seems unlikely that Ca^{++} is involved in the generation of the hypothetical second signal for B-cell activation (Dukor and Hartmann, 1973). This fact, and the inability of Mg^{++} to restore the immune response in EGTA-treated cultures, argues strongly against any involvement of activated C3 in the signal(s) generation either at the level of B cells or at the level of macrophages.

Fig. 7. illustrates the Ca^{++}-dependent and Ca^{++}-independent periods during the immune response.

The antibody-forming precursor cell bearing specific receptors for the antigen (B_1) proliferates after the lag period (B_1L) to B_2–B_K–B_{n-1} cells. The lag period is independent of the presence of exogeneous free Ca^{++}. However, the whole, or perhaps one critical cell division (Nakamura, Segal, Globerson and Feldman, 1972), can take place only in the presence of Ca^{++}. The differentiation step(s) leading to the antibody-forming cells is inhibited by Ca^{++}.

The situation is, however, more complex, since SRBC is a T-cell dependent antigen. Therefore the possibility that T cells or their function(s) might be influenced by Ca^{++} cannot be excluded. It can be assumed that in a spleen cell population derived from antigen-primed mice the number of B_1 cells is smaller than in non-primed spleens and the number of B memory cells is increased. In addition, we have to assume that in this case proliferative and differentiation steps leading to antibody-forming cells would be more overlapping than in the primary response. Therefore it is not surprising that removal of Ca^{++} from the cultures during such a period does not completely inhibit the secondary immune response. Since Ca^{++} acts antagonistically on cell proliferation and cell differentiation, processes which take place simultaneously in the secondary immune response, addition of Ca^{++} to EGTA-containing cultures at any time after antigenic stimulation enhances but does not completely restore the immune response. The degree of the enhancement of the immune response by Ca^{++} in this case would be dependent on the proportion of the B cells in their various stages of maturation present in the spleen cell population of primed mice.

The finding that removal of Ca^{++} from the cultures by EGTA increases and addition of Ca^{++} decreases the ratio of 7S to 19S PFC supports the suggestion that cell differentiation is controlled by Ca^{++}. However, the interpretation of these results is difficult. Data available at present do not permit us to distinguish between at least two possibilities: (1) whether EGTA influences the switch from 19S to 7S PFCs; or (2) whether the differential effect of EGTA on the triggering of differentiation of 7S precursors to 7S PFC versus 19S precursors to 19S PFC is responsible for the results obtained. Since the 7S response is T cell-dependent the possibility cannot be excluded that Ca^{++} acts on these cells or that the effect of T cells is mediated by Ca^{++}-dependent processes.

We should like to postulate that Ca^{++} generally controls cell division and cell differentiation. The antibody formation *in vitro* seems to be a suitable model for investigation of factors controlling these processes.

Both site and mechanism of action of Ca^{++} for regulation of these processes is still unknown. Probably one of the most fascinating possibilities is its relation to the action of cyclic AMP and cyclic GMP (Rasmussen, 1970; Rasmussen and Nagata, 1970; Whitfield, MacManus, Youdula and Franks, 1971: Whitfield, MacManus and Gillan, 1973). It seems possible that Ca^{++} either regulates the intracellular level of these nucleotides, which have been reported to act antagonistically in various biological systems (Hadden, Hadden, Hadox and Goldberg, 1972; Goldberg, O'Dea and Haddox, 1973;

Goldberg, Haddox, Dunham, Lopez and Hadden, 1973; Estensen, Hill, Quie and Hogan, 1973) and/or that its action is mediated by such Ca^{++}-controlled mechanism.

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