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THE EFFECT OF FREUND'S COMPLETE ADJUVANT ON THE CELLULAR RESPONSE IN MICE TO SHEEP ERYTHROCYTES

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SUMMARY

The effect of Freund's complete adjuvant on the cellular response in BALB/c mice to SRBC was studied using techniques based on immunocytoadherence (ICA), inhibition of ICA using an antiserum to the theta alloantigen, and immune adherence (IA). Particular attention was paid to the cellular morphology of the responding lymph nodes, details of which are described.

INTRODUCTION

Immunological adjuvants may be divided into two main categories: those which provide a direct, non-specific proliferative stimulus to the reticuloendothelial system (White, Coons & Connolly, 1955) and those which retain the antigen in a depot at the site of inoculation (Halbert, Mudd & Smolens, 1946). The latter includes Freund's complete adjuvant (FCA), which consists of a stable water-in-oil emulsion containing heat-killed Mycobacteria. Localized administration of this with antigen results in enhanced and prolonged antibody formation and a marked increase in the cell population of the draining lymph node (Ehrich *et al.*, 1945; Freund, 1947). Moore *et al.* (1963) studied the cellular changes in the spleen and lymph nodes, resulting from intravenous administration of FCA with diphtheria toxoid, and the relationship of these changes to the humoral response. The cellular developments resembled those of a classic primary response with immediate progression to a secondary response; no particular cell type appeared to predominate at any stage.

This paper describes quantitative and morphological studies of responding lymph node cells in BALB/c mice stimulated with sheep erythrocytes (SRBC) or SRBC + FCA, based on immunocytoadherence (ICA) studies (Nota *et al.*, 1964; Zaalberg, 1964; Duffus & Allan, 1969, 1971); rosette inhibition tests using a heterologous antiserum to the theta (θ) alloantigen of BALB/c mice; and an immune adherence (IA) technique modified to identify θ-bearing cells, with an indicator system of human erythrocytes (HRBC) and guinea-pig complement (Turk, 1958; Melief *et al.*, 1967; Forget *et al.*, 1970; Allan & Rapson, 1974).

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MATERIALS AND METHODS

Animals

Carnworth inbred BALB/c mice weighing approximately 25 g were used in these experiments.

Antigen

Sheep erythrocytes (SRBC) used for both immunization and *in vitro* assays were obtained from a Clun Forest Ewe. They were washed four times in 0·85 per cent saline before use.

Adjuvant

Freund's complete adjuvant was obtained from Difco Laboratories.

Rabbit antiserum to BALB/c brain-associated theta alloantigen (anti-BA θ antiserum)

This was prepared according to the method of Golub (1971), and at a dilution of 1:16 gave 90, 44 and 1 per cent cytotoxicity against BALB/c thymocytes, lymph node cells and bone marrow cells respectively.

Experimental procedure

Mice were injected subcutaneously on the left shoulder with 4×10^8 SRBC administered either in saline (group SC) or emulsified with FCA (group SCA). On days 2–10, 15, 20 and 25–40 after inoculation, five animals from each group were killed; a further group of five animals served as an uninoculated control. The left (draining) axillary lymph node was removed and immediately placed in Parker's TC 199 medium (Flow Laboratories) supplemented to 10 per cent with foetal bovine serum (Flow Laboratories).

Preparation of lymph node cell suspensions

Each lymph node was teased apart and further dispersed through a fine bore Pasteur pipette. The resulting suspension was filtered through stainless steel gauze of 35 μm 'pore' size and washed twice in ice-cold medium. A total cell count was made and the concentration adjusted to 1×10^7 lymph node cells/ml.

These cell suspensions were used to investigate the effect of FCA on the cellular response using techniques based on immunocytoadherence (ICA), rosette inhibition (RI) using anti-BA θ antiserum, and immune adherence (IA). The cellular morphology of the responding lymph node was studied in each experiment. These techniques are fully described in the Results section.

RESULTS

Immunocytoadherence (ICA) technique

The method used was similar to that described by Duffus & Allan (1969, 1971). SRBC were added to each lymph node cell suspension to give a 30:1 ratio of SRBC to lymph node cells. The mixtures were incubated at 37°C for 2 hr, being shaken at 15-min intervals. After incubation samples were diluted to 1:10 and the numbers of ICA rosettes counted in a haemocytometer. Cells with a minimum of five adhering erythrocytes were scored as rosettes. Between 7×10^4 and 4×10^6 lymph node cells were scanned and the number of rosette-forming cells (RFC) per lymph node calculated.

Differential counts

Samples containing rosettes were centrifuged directly on to microscope slides in a cytocentrifuge (Shandon) at 80 *g* for 5 min. The resulting monolayers were fixed and stained with Triple Leishman Stain. A differential assessment of morphology based on fifty RFC was made by light microscopy according to the criteria defined by Duffus & Allan (1971).

The results of the ICA studies are shown in Figs 1, 2, 3a and 3b. A comparison of total RFC per lymph node, resulting from localized administration of SRBC+FCA, is illustrated in Fig. 1. The group of mice receiving SRBC alone (SC) were given a secondary challenge on day 25; thereafter a striking similarity was observed between the secondary response in this group and the increase in RFC which occurred naturally in SCA. The total RFC counts were higher in SCA, peaking initially on day 8 and again on day 30.

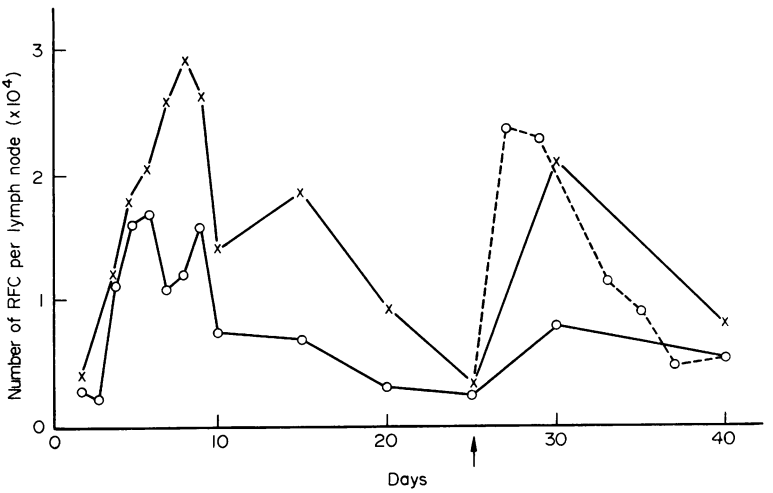

FIG. 1. Total immunocytoadherence (ICA) rosette-forming cells (RFC) per lymph node. (o—o) Group of mice inoculated with SRBC alone (SC). (×—×) Group of mice inoculated with SRBC+FCA (SCA). (o- - -o) Anamnestic response to a secondary challenge with SRBC.

Fig. 2 shows the total lymph node cell counts in both groups; cell populations in SCA nodes were the greater throughout the response. Peaks occurred on days 7 and 30 corresponding to the RFC peaks.

Fig. 3a and b show the percentages of large and small blast RFC, respectively, in both groups of mice. The numbers of large blasts were markedly increased in SCA, particularly on days 5, 20 and 40, whereas in SC a peak occurred on day 5 only. However, following the secondary challenge to SC, a marked anamnestic response of large blasts was seen, reaching a peak on day 29. Small blast counts were similar in both groups, peaking at days 9 and 30; again, after secondary challenge a sharp increase occurred in SC reaching a peak on day 33, i.e. after the large blast peak.

Rosette inhibition (RI) test

Rosette inhibition tests using anti-BA θ antiserum were performed on days 4, 5 and 9 at 37°C and on day 9 at 4°C. Before incubation with SRBC each lymph node node cell suspension was divided into two aliquots and incubated with either anti-BA θ antiserum or normal

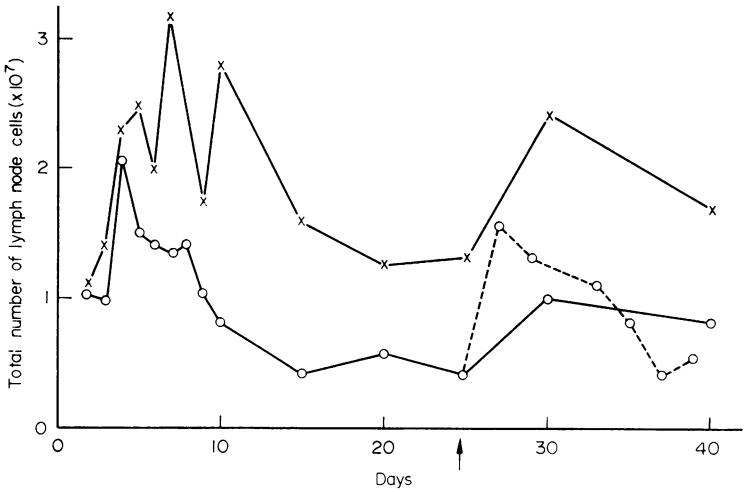

FIG. 2. Total lymph node cell populations. (○—○) Group of mice inoculated with SRBC alone (SC). (×—×) Group of mice inoculated with SRBC+FCA (SCA). (○- - -○) Anamnestic response to a secondary challenge with SRBC.

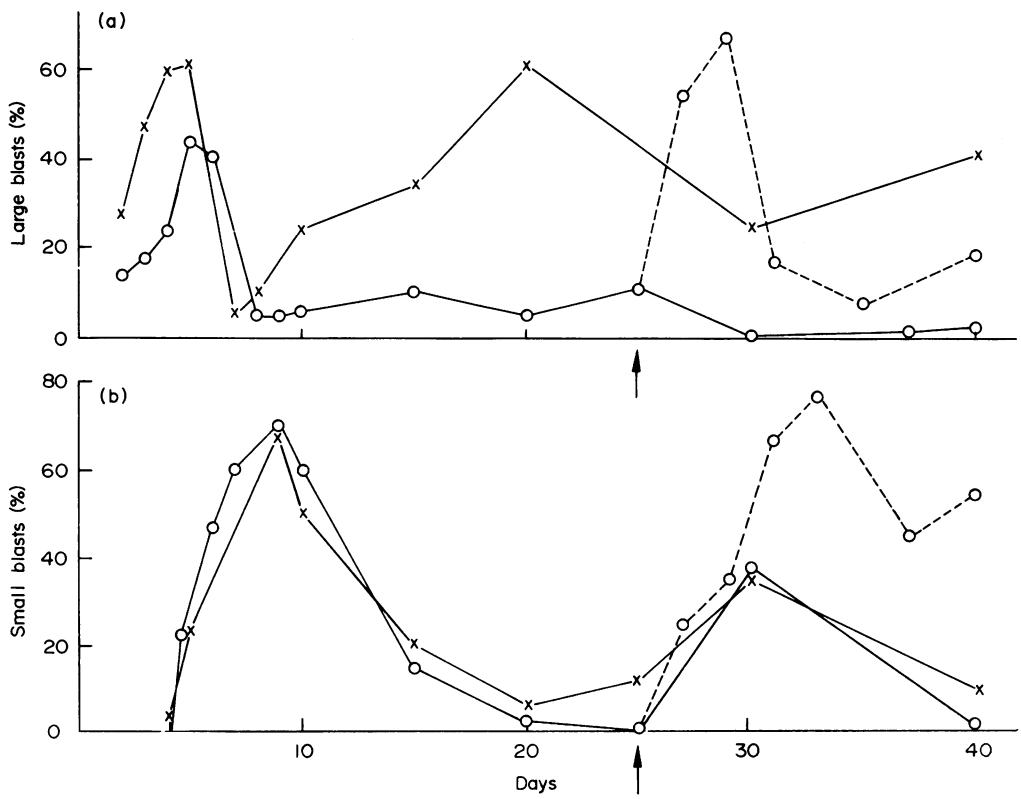

FIG. 3. (a) Percentage values of ICA rosette-forming large blasts. (b) Percentage values of ICA rosettes forming small blasts. (○—○) Group of mice inoculated with SRBC alone (SC). (×—×) Group of mice inoculated with SRBC+FCA (SCA). (○- - -○) Anamnestic response to a secondary challenge with SRBC.

rabbit serum (NRS) for 30 min at the required temperature. The cells were washed once in ice-cold medium and treated as described in the ICA technique. Differential counts were made as previously described.

The total inhibition of ICA expressed as percentages of total cell counts showed that on days 4, 5 and 9 there were 50, 40 and 40 per cent more θ-bearing cells present in SCA than in SC.

Fig. 4a–f illustrates the counts of morphologically differing cells forming rosettes after pre-incubation with NRS or anti-BA θ antiserum: the only cell type to show inhibition was

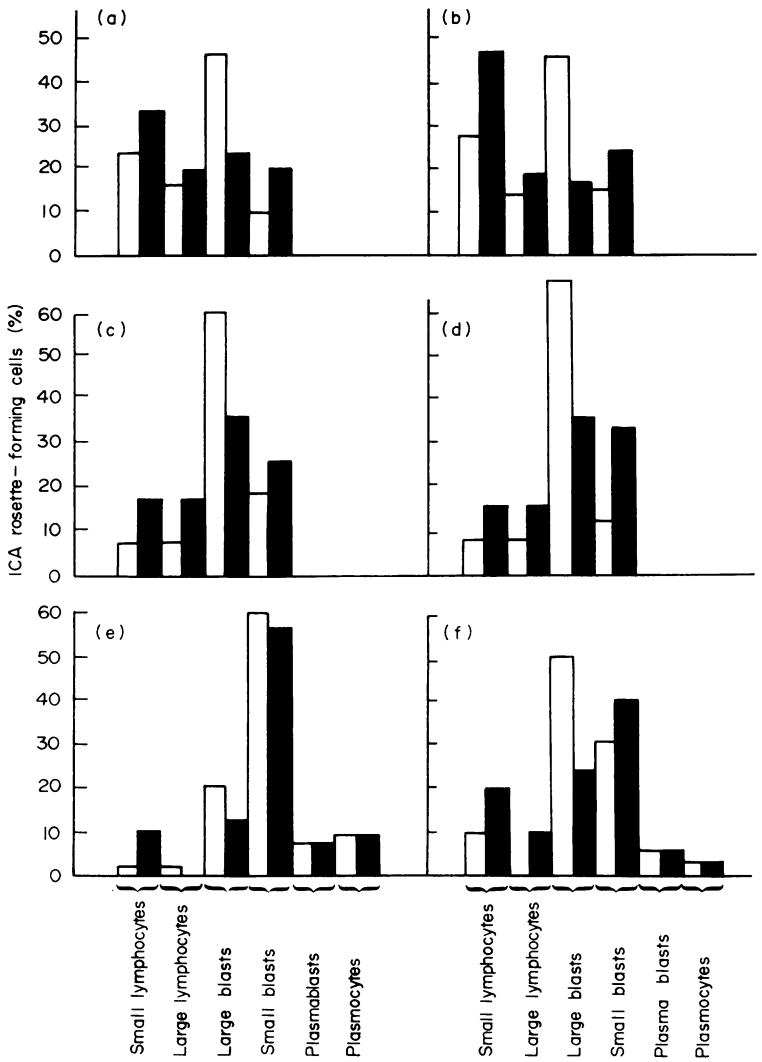

FIG. 4. Morphological differentiation of rosette-forming cells after preincubation with either normal rabbit serum or anti-BA θ antiserum.

(□) Preincubation with normal rabbit serum. (■) Preincubation with anti-BA θ antiserum. (a), (c) and (e) SC. (b), (d) and (f) SCA. (a) and (b) Day 4. (c) and (d) Day 5. (e) and (f) Day 9.

the large blast. On days 4, 5 and 9 there was 66, 41 and 40 per cent inhibition respectively of large blasts in SC; in SCA these values were 68, 49 and 56 per cent; greater inhibition of large blast rosette formation occurred in SCA than in SC and was particularly notable 9 days after immunization.

Since the differential counts of RFC for morphological assessment were based on fifty RFC per sample, any decrease in the percentage of large blasts will be offset by an increase in the remaining cell types. There was only one RFC type (large blast) inhibited at 37°C, so this decrease was judged to be significant.

No visible inhibition of small blasts occurred at any time, despite the high numbers of these cells known to be forming rosettes in both groups on days 4 to 9 (see Fig. 3b). Earlier experiments in this laboratory (Duffus & Allan, 1971; Elson *et al.*, 1972) have shown that small blasts form large rosettes at 37°C by secreting antibody, so that any surface antigens on these cells may be concealed by anti-SRBC immunoglobulin.

When the RI test was repeated at 4°C, a temperature at which cellular secretion of antibody does not occur and rosettes are only single layered (Elson *et al.*, 1972), the percentages of rosette-forming small blasts were lower than at 37°C. Therefore some small blasts do appear to bear the θ alloantigen.

Immune adherence (IA) technique

This was performed on days 4, 5 and 9 after immunization. The technique used was based on that described by Forget *et al.* (1970). Lymph node cell suspensions were prepared as described above, in gelatin veronal buffer and the concentration adjusted to 1×10^7 lymph node cells/ml. To 0·5 ml of each suspension an equal volume of 1:32 dilution of anti-BA θ antiserum was added, making a final dilution of 1:64. After incubation at 37°C for 30 min the cells were washed once in buffer and resuspended to a concentration of 1×10^7/ml. After preliminary parameter experiments, guinea-pig complement was added to a final concentration of 2 per cent. Group O HRBC were added to give a 30:1 ratio of HRBC to lymph node cells and the mixture incubated at room temperature for 75 min, being carefully inverted at 15-min intervals. Samples diluted to 1:10 were examined in a haemocytometer and the numbers of IA rosettes were counted in the four major corners of the grid. Cells with a minimum of three adhering HRBC were scored as rosettes. The number of lymph node cells was counted over a similar area at a dilution of 1:20 and the percentage of θ-bearing cells calculated. Differential counts were again made.

TABLE 1. Percentages of total θ-bearing cells, both non-responding and responding to SRBC (IA technique)

Day	Group	Total population of θ-bearing cells per lymph node	Approximate percentage of total lymph node cell population
4	SC	$0{\cdot}37 \times 10^7$	30
	SCA	$0{\cdot}39 \times 10^7$	44
5	SC	$0{\cdot}26 \times 10^7$	21
	SCA	$0{\cdot}30 \times 10^7$	28
9	SC	$0{\cdot}12 \times 10^7$	20
	SCA	$0{\cdot}38 \times 10^7$	25

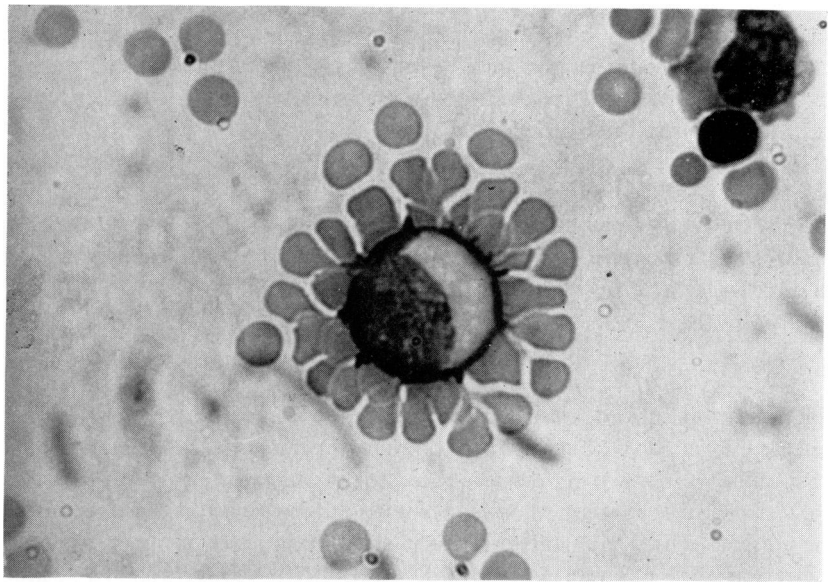

FIG. 5. A typical θ-bearing large blast, coated with anti-BA θ antiserum and incubated with human erythrocytes in the presence of complement. (Immune adherence.)

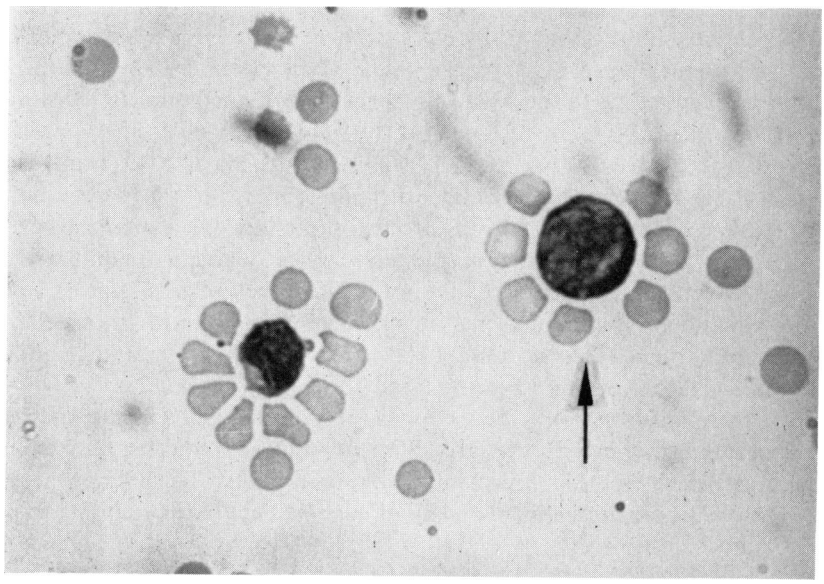

FIG. 6. A typical θ-bearing small blast (arrowed), coated with anti-BA θ antiserum and incubated with human erythrocytes in the presence of complement. (Immune adherence.) The smaller cell is a θ-bearing lymphocyte.

Table 1 shows the percentage of θ-bearing cells as determined by the IA technique, i.e. cells which are θ-bearing but are not necessarily responding to SRBC. On days 4, 5 and 9 the increases in the percentages of θ-bearing cells in SCA over those in SC were 50, 50 and 25 per cent respectively. IA differential counts were corrected to control results obtained from unresponding lymph nodes.

The percentages of θ-bearing, responding large blasts (illustrated in Fig. 5) on days 4, 5 and 9 were 57, 60 and 125 per cent greater in SCA than in SC. Theta-bearing small blasts (illustrated in Fig. 6) showed lower percentages in SCA on each of these days. It was further noted that θ-bearing lymphocytes became progressively less numerous due, perhaps, to transformation to blast cells.

DISCUSSION

Lymph node cell populations increased in SCA as shown in Fig. 2. That this increase was antigen selective is shown in Fig. 1. In SCA, an enhancement of ICA–RFC occurred compared to SC and this increase partly accounted for the increase in lymph node cell population. The question arises as to whether an increased cellularity *per se* presents more lymphoid cells to the antigen (which in turn is trapped in the lymph node) or a positive antigen-sensitive cell selection occurs. Taub, Krantz & Dresser (1970) showed that FCA alone caused marked enlargement of the draining lymph nodes of CBA mice 4 days after localized inoculation; paracortical hyperplasia and hypercellularity accompanied by blast transformation were also observed. This was followed by germinal centre hyperplasia and medullary plasmocytosis occurring between days 4 and 10. It was suggested that paracortical expansion induced by adjuvant was partly due to an augmented cellular traffic with a net influx of recirculating lymphocytes into these areas.

Differential counts showed that in SCA, antigen-sensitive large blasts were present in greater numbers than those in SC throughout the 40-day period after immunization, the peak on day 4 corresponding to the blast transformation of uncommitted cells observed by Taub *et al.* (1970). It is of interest to note that the only RFC type to show inhibition with anti-BA θ antiserum in the RI test at 37°C was the large blast. Also, transformation of lymphocytes to large blasts following antigen stimulation has obviously not resulted in the loss of θ. RI tests comparing both groups of mice show that the aforementioned increase in cell population in SCA can be partly accounted for by an increase in number of θ-bearing large blasts, i.e. T cells. RI tests at 37°C did not indicate the presence of θ on small blasts; however, the alloantigen can be detected at 4°C on the surfaces of these cells, which at 37°C may secrete antibody (Elson *et al.*, 1972). The increase in the number of θ-bearing small blasts followed that of the θ-bearing large blasts.

The observed similarity between the secondary response in SC and the late response in SCA, with particular reference to the large blast population, indicates that the secondary antigen stimulus simulates the adjuvant by invoking a greater T-cell response. Allison & Davies (1971) have demonstrated increased T-cell involvement in the response to immunization with FCA.

Unlike the preceding tests, the IA technique will positively identify a θ-bearing cell. Furthermore, it picks up both responding and non-responding T cells. As shown in Table 1, FCA, when incorporated into the immunizing preparation, produces a very marked increase in the numbers of T cells in the draining lymph node; this suggests an increased response on

the part of the T-cell population, in which case a large percentage of these cells would be expected to respond to SRBC. Indeed, in SCA, greater percentages of antigen-sensitive T cells (large blasts) were recorded, viz: 57, 60 and 125 per cent greater on days 4, 5 and 9 respectively, than in SC.

The continued presence of θ alloantigen on the surface of T cells makes it possible to monitor their response and to calculate the numbers of lymph node cells responding and not responding to the immunizing antigen. Data from the comparison of the effects of FCA alone (Taub *et al.*, 1970), antigen alone and antigen+FCA on draining lymph node cell populations strongly suggests that FCA not only increases numbers of lymphocytes, but, by inducing blast transformation, promotes more efficient antigen uptake with consequent enhancement of the T-cell response.

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