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# Rosette-forming Cells and the Immunological Response after DNCB, DNP-carrier and Oxazolone Sensitization

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**Summary.** Guinea-pigs have been sensitized with DNCB, DNP-guinea-pig serum and DNP-guinea-pig erythrocytes. Four, six and ten days after sensitization numbers of rosette-forming cells with DNP-erythrocytes were estimated in the draining lymph nodes.

It was found that the numbers of DNP-erythrocyte RFC did not correlate with humoral immunity as estimated by haemagglutination assay, but did so with cell-mediated immunity estimated as delayed-type hypersensitivity. On the other hand histological examination of the draining lymph nodes gave no clear confirmation of such a relationship.

Experiments on the antigenic specificity of RFC showed that after DNCB sensitization a clear increase in oxazolone-erythrocyte RFC and SRBC RFC can be found. These latter data suggest formation of RFC to be at least partly a non-specific phenomenon.

## INTRODUCTION

In recent years many reports dealing with the relation of antigen-specific rosette-forming cells (RFC) to B and T lymphocytes have appeared. The conclusions obtained are often contradictory. Many authors conclude that mainly B cells participate in the formation of RFC (Gorzynski, Miller and Philips, 1971; Hunter, Munro and McConnell, 1972; Takahashi, Old, McIntire and Boyse, 1971). Comparable results have been obtained by Roberts, Brandriss and Vaughan (1971), who could not demonstrate a correlation between the number of RFC and the presence of delayed type hypersensitivity.

In contrast, Hogg and Greaves (1972), Bach and Dardenne (1972), Argyris, Haritou and Cooney (1972), and Wilson and Miller (1971), in experiments using anti-T cell sera, found that RFC were at least partially composed of T cells. It was later discovered that temperature is an important factor: RFC prepared at low temperature (0–4°) contain relatively more T lymphocytes than after preparation at 37° (Elson, Allan, Elson and Duffus, 1972; Cone and Wilson, 1972).

In the studies to be described the relationship between the number of RFC prepared at low temperature and different ratios of cell-mediated immunity (CMI) to humoral immunity has been investigated. Groups of guinea-pigs have been sensitized to 2,4-dinitrochlorobenzene (DNCB) and to different DNP-carriers. The level of CMI was estimated by measuring the increase in paracortical area in draining lymph nodes and skin testing the animals. The level of humoral response was estimated by counting the number of active

germinal centres in the lymph nodes and by antibody assay in the serum. Specificity of RFC was investigated by estimation of the numbers of SRBC RFC and oxazolone erythrocyte RFC after sensitization by contact with DNCB and oxazolone.

## MATERIALS AND METHODS

*Animals*

Female albino guinea-pigs, weighing 250–350 grams, have been used.

*DNP-erythrocytes*

Allogeneic guinea-pig erythrocytes were prepared from citrated blood by washing the blood cells twice with buffered saline and then twice with a basic phosphate solution (0·02 M $Na_2HPO_4$, 0·10 M NaCl, pH 8·0). A washed erythrocyte pellet suspension was combined with at least eight volumes of the basic phosphate solution saturated with 2,4-dinitro-fluorobenzene (DNFB) and shaken gently for 15 minutes at room temperature. Immediately afterwards the conjugated red cells were washed and stored for a maximum of 2 days in phosphate-buffered glucose (PBG, 0·057 M $Na_2HPO_4$, 0·018 M $KH_2PO_4$, 0·15 M glucose).

*DNP-erythrocyte ghosts*

A DNP-erythrocyte pellet suspension was lysed by a 20-fold volume of phosphate–EDTA buffer, according to Zwaal and van Deenen (1968), and cleared from remaining intact cells by centrifuging (10 minutes, 2000 ***g***). The DNP-ghosts were washed (20 minutes, 30,000 ***g***) several times to remove free haemoglobin.

*DNP-guinea-pig serum*

Forty milligrams of DNFB were added to 20 ml pooled guinea-pig serum. The reaction mixture was kept at pH 7·5 (by adding 1 N NaOH) and stirred for 5 hours at room temperature.

*Oxazolone-erythrocytes*

Oxazolone-guinea-pig erythrocytes were prepared according to Askenase and Asherson (1972).

*Sensitization*

Groups of guinea-pigs were sensitized either by epicutaneous application of 0·15 ml of a 4 per cent (w/v) solution of DNCB in 96 per cent ethanol or 0·15 ml 10 per cent oxazolone (4-ethoxymethylene-2-phenol oxazolone) in 96 per cent ethanol (warm) on both ears, or by injecting 0·1 ml ($10^8$) DNP-erythrocytes or 0·075 ml DNP-guinea-pig serum into both ears. As controls guinea-pigs were injected with unconjugated erythrocytes or guinea-pig serum. No significant differences between the values obtained from these two control groups were found for any of the parameters used. This made us decide to pool the control values, obtained in each test from almost equal numbers of both control groups.

*Preparation of lymphocyte suspensions and rosette testing*

Lymph node and spleen cell suspensions were prepared by teasing the tissues in Hanks's

balanced salt solution. The suspensions were filtered through a cotton wool filter and washed twice.

The percentage of immunoblasts was estimated in the cell suspensions by counting them on slides prepared in a cytocentrifuge and stained with Leishman stain. Total numbers of blast cells were calculated by multiplying percentages by $5{\cdot}5 \times 10^5$, the mean number of lymphocytes per mg lymph node weight (Meyer, 1971), and the corresponding mean lymph node weight.

Rosette-forming cells were prepared according to McConnell, Munro, Gurner and Coombs (1969) with slight modifications. During the preparation of the rosettes cells were kept in melting ice. The rosette suspension was prepared in PBG–75 per cent foetal calf serum (FCS). At least $10^5$ white cells were scanned. Total numbers of RFC were calculated as described for total numbers of blast cells

*Histology*

Cervical lymph nodes, draining the site of sensitization, were fixed in Carnoy's solution and sectioned semi-serially at 5 $\mu$m. The mean size of the paracortical area and the mean number of active germinal centres per section was estimated by examination of sections from five different levels.

*Haemagglutination assay*

Antibody titres were determined by using DNP-erythrocytes and two-fold serial serum dilutions made with PBG–10 per cent FCS.

*Skin-testing*

Skin tests were performed 10–12 days after sensitization either by epicutaneous painting with 20 $\mu$l of a 0·4 per cent DNCB solution in petroleum ether (boiling range 60–80°): olive oil (9:1) (Baumgarten and Wilhelm, 1969), or by intracutaneous injection of 0·1 ml ($10^8$) DNP-erythrocytes or 500 $\mu$g DNP-erythrocyte ghosts. Induration of the skin reactions produced was assessed by measuring double skin thicknesses in and at two sides of the test sites. Relative indurations were calculated by subtracting at random the induration found in guinea-pigs injected with unconjugated erythrocytes or erythrocyte ghosts from the induration in the test groups.

## RESULTS

### SPLEEN WEIGHTS AND WEIGHTS OF LYMPH NODES DRAINING THE SENSITIZATION SITE

The increase in weights of the auricular, cervical and tracheal lymph nodes and the spleen after sensitization with different DNP-carriers is presented in Figs 1 and 2. From these figures it is clear that after DNCB painting there is a marked increase in lymph node weights. As could be expected this increase is more marked in the auricular and cervial nodes. The other antigens only cause a slight reaction.

### BLAST CELL COUNTING AND HISTOLOGICAL EXAMINATION OF A DRAINING LYMPH NODE

The total number of blast cells in the cervical nodes 4, 6 and 10 days after sensitization is presented in Fig. 3. After DNCB painting a marked increased number at 4 days slowly decreases to nearly the control values at 10 days after painting. DNP-guinea-pig serum

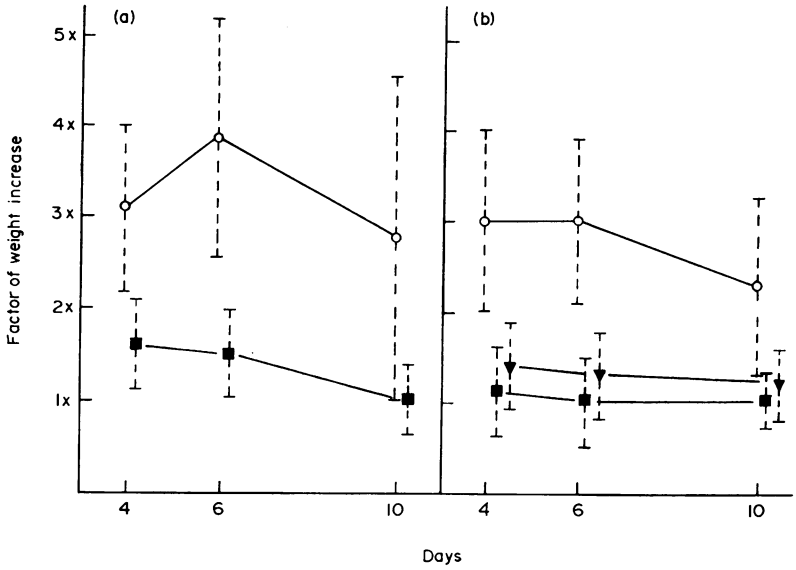


FIG. 1. Weight increase factors (weights at random divided by control weights) for (a) the auricular and (b) cervical lymph nodes after sensitization with (○) DNCB, (■) DNP-erythrocytes and (▼) DNP-guinea-pig serum. The mean and S.D. for each point were obtained from at least eight values.

injection causes a slight increase over the controls. The mean paracortical surface area at 4 and 6 days after sensitization and the number of germinal centres at 10 days are presented in Table 1. It seems, that a significant increase in paracortical surface area and number of active germinal centres compared to the control values can only be observed after DNCB painting (Wilcoxon, $P = 0{\cdot}005$).

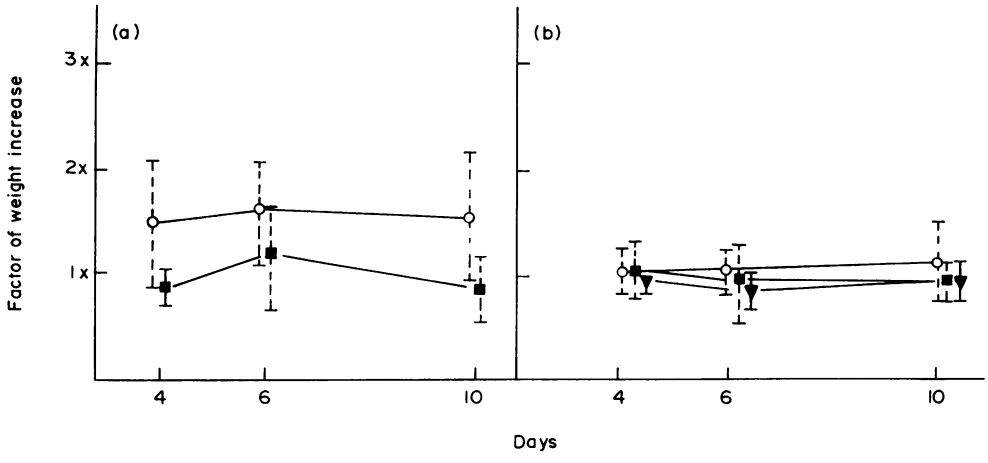


FIG. 2. Weight increase factors (weights at random divided by control weights) for (a) the tracheal lymph node, and (b) the spleen after sensitization with (○) DNCB, (■) DNP-erythrocytes and (▼) DNP-guinea-pig serum. The mean and S.D. for each point were obtained from at least eight values.

ROSETTE-FORMING CELLS

Fig. 4 shows the numbers of RFC per thousand as estimated with DNP-erythrocytes in different lymph nodes and the spleen. There is an increase in RFC after DNCB painting and injection of DNP-erythrocytes. This increase is more evident in the former groups of

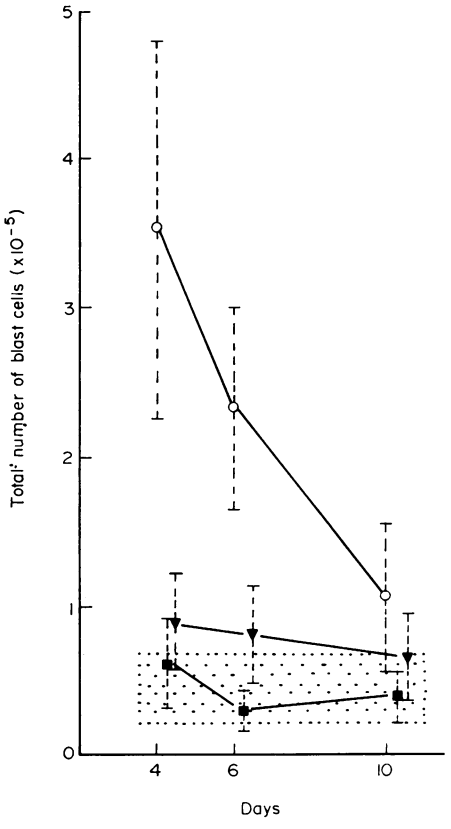


FIG. 3. Total number of blast cells per cervical lymph node after sensitization with (○) DNCB, (■) DNP-erythrocytes, and (▼) DNP-guinea-pig serum. The mean and S.D. for each point were obtained from four values. The dotted area represents the mean and S.D. for the controls.

TABLE 1

QUANTITATIVE HISTOLOGICAL DATA * OBTAINED FROM CERVICAL LYMPH NODES

| | Sensitization | | | |
|---|---|---|---|---|
| | DNCB | DNP-guinea-pig serum | DNP-erythrocytes | Controls |
| Paracortical surface area† | 4 days 154±45<br>6 days 237±98 | 96±41<br>109±16 | 97±16<br>116±51 | 83±11 |
| Germinal centres ‡ | 10 days 15±4 | 6± 5 | 4± 2 | 3± 2 |

* Each value represents three lymph nodes.
† Arbitrary units.
‡ Mean number of germinal centres per section.

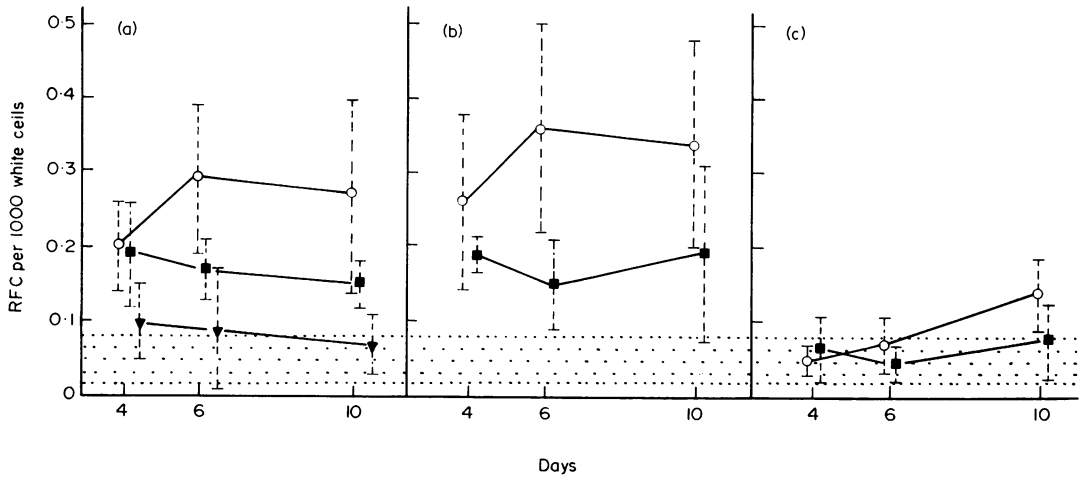


FIG. 4. Numbers of DNP-erythrocyte RFC per 1000 white cells in (a) the cervical lymph nodes, (b) pooled auricular, cervical and tracheal lymph nodes and (c) the spleen after sensitization with (○) DNCB, (■) DNP-erythrocytes, and (▼) DNP-guinea-pig serum. The mean and S.D. for each point were obtained from at least four values. The dotted area represents the mean and S.D. for the controls.

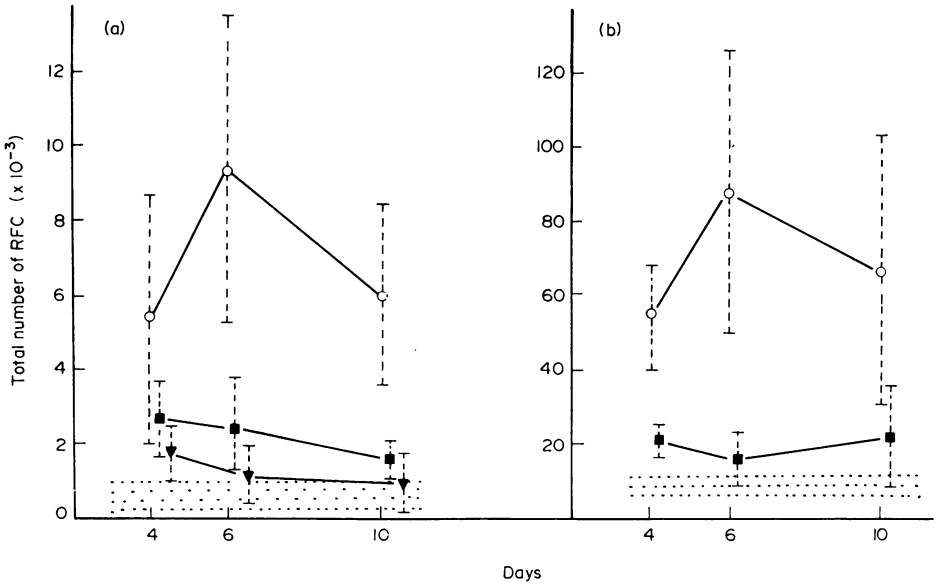


FIG. 5. Total number of DNP-erythrocyte RFC in (a) one cervical lymph node and (b) in six pooled draining lymph nodes (auricular, cervical and tracheal) after sensitization with (○) DNCB, (■) DNP-erythrocytes, and (▼) DNP-guinea-pig serum. The mean and S.D. for each point were obtained from at least four values. The dotted area represents the mean and S.D. for the controls.

sensitized animals. The spleens showed an indication of increase of the number of RFC at 10 days after DNCB painting. The DNCB-painted guinea-pigs contained even more RFC when expressed as a total number (Fig. 5).

In order to investigate the specificity of the increase in RFC found, some control

TABLE 2

EXPERIMENTS ON THE ANTIGENIC SPECIFICITY OF RFC. NUMBERS OF RFC* PER 1000 CERVICAL LYMPH NODE CELLS AS PREPARED WITH DNP-GUINEA-PIG ERYTHROCYTES AND OXAZOLONE-GUINEA-PIG ERYTHROCYTES 6 DAYS AFTER SENSITIZATION WITH DNCB AND OXAZOLONE

| Sensitization | Cervical lymph node weight (mg) † | RFC per 1000 (prepared with DNP-erythrocytes) | RFC per 1000 (prepared with oxazolone erythrocytes) |
|---|---|---|---|
| DNCB | 49·0 ± 20·3 | 0·25 ± 0·07 | 0·78 ± 0·22 |
| Oxazolone | 26·7 ± 6·5 | 0·06 ± 0·03 | 1·21 ± 0·81 |
| Not sensitized | 21·3 ± 5·6 | 0·06 ± 0·02 | 0·31 ± 0·08 |

* Mean and S.D. obtained from five cell suspensions (five guinea-pigs).
† Each value represents ten lymph nodes.

experiments were done. In animals sensitized to oxazolone or DNCB after 6 days RFC were prepared to DNP-erythrocytes and oxazolone-erythrocytes. The results are presented in Table 2. From this table it is clear that an increase in the number of DNP-erythrocyte RFC can be found after DNCB sensitization and not after oxazolone sensitization.

The number of oxazolone-erythrocyte RFC however, is not only increased after sensitization by contact with oxazolone but also after DNCB-sensitization. There is generally a difference between the numbers of DNP-erythrocyte RFC and oxazolone-erythrocyte RFC, the latter being higher. This is also true for the non-sensitized nodes. In Table 3 the number of RFC as prepared with sheep red cells are presented. A clear increase of SRBC RFC after DNCB sensitization can be observed. The numbers of SRBC RFC even in control animals are high compared to the numbers of DNP-erythrocyte RFC (Table 2).

TABLE 3

EXPERIMENTS ON THE ANTIGENIC SPECIFICITY OF RFC. NUMBERS OF SHEEP RED CELL RFC * PER 1000 CERVICAL LYMPH NODE CELLS 6 DAYS AFTER SENSITIZATION WITH DNCB

| Sensitization | Cervical lymph node weight (mg) † | Percentage RFC (prepared with SRBC) |
|---|---|---|
| DNCB | 57·9 ± 20·0 | 1·47 ± 0·50 |
| Not sensitized | 17·5 ± 4·1 | 0·45 ± 0·15 |

* Mean and S.D. obtained from five cell suspensions (five guinea-pigs).
† Each value represents ten lymph nodes.

### HAEMAGGLUTINATION ASSAY

The antibody titres as measured with DNP-erythrocytes at different intervals after sensitization are presented in Fig. 6. The increase in titre, which starts at 6 days, is in general low, but in all sensitized groups it is significant. Injection of DNP-erythrocytes gives the most marked increase, while DNCB painting tends to give a slightly less obvious response.

### SKIN-TESTING

Table 4 presents the results of skin testing the DNCB-painted and DNP-erythrocyte-injected animals with DNCB. Skin thickness is significantly increased after 48 hours in

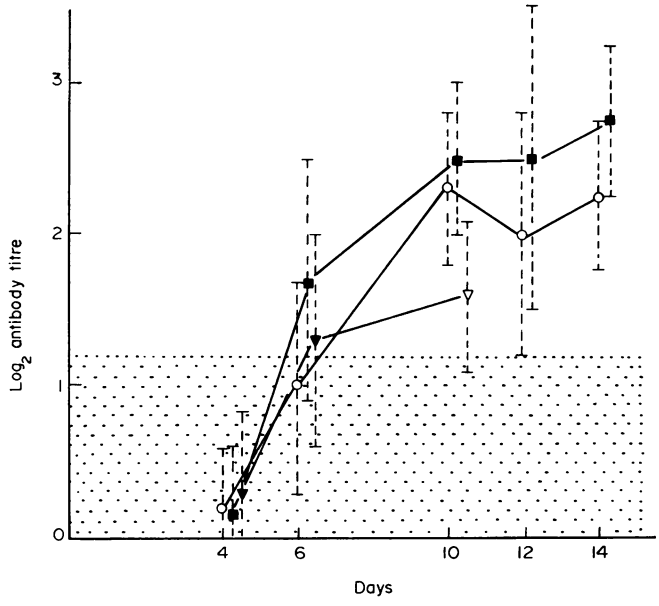


FIG. 6. Haemagglutination titres as determined with DNP-erythrocytes after sensitization with (○) DNCB, (■) DNP-erythrocytes and (▼) DNP-guinea-pig serum. The values for 6 and 10 days after sensitization represent ten guinea-pigs each point, the other values six each point. The dotted area represents the mean and S.D. for the controls.

the groups sensitized by painting with DNCB. Also erythema, not presented in the Table, was only found at 24 and 48 hours in the group of animals sensitized by painting with DNCB.

Fig. 7 shows the results of skin-testing the same groups of animals with DNP-erythrocytes. As a group the data obtained for testing at 24 and 48 hours after sensitization by painting with DNCB are significantly higher than those obtained in animals sensitized by injection of DNP-erythrocytes (Wilcoxon, $P = 0{\cdot}001$). Erythema was in general difficult to interpret. Skin-testing with DNP-ghosts produced comparable results, which are however not significant ($P = 0{\cdot}04$, Fig. 8).

TABLE 4

SKIN TESTING WITH DNCB. RELATIVE INDURATIONS WERE CALCULATED BY SUBTRACTING AT RANDOM THE INDURATION FOUND IN GUINEA-PIGS INJECTED WITH UNCONJUGATED ERYTHROCYTES FROM THE INDURATION IN THE TEST GROUPS. EACH VALUE REPRESENTS NINE ANIMALS (ONE TEST SITE PER ANIMAL)

| Sensitization | 24 hours | 48 hours |
|---|---|---|
| DNCB | 0·14±0·28 mm | 0·54±0·39 |
| DNP-erythrocytes | 0·04±0·14 | 0·06±0·21 |

## DISCUSSION

The main purpose of this investigation was to correlate numbers of RFC with cell-mediated or humoral immunity. In order to get different ratios of CMI and humoral

immunity we used three sensitization procedures, namely painting with DNCB and injection of either DNP-erythrocytes or DNP-guinea-pig serum. From the results of the study of the reaction in the regional lymph nodes, as shown in Figs 1 and 2 (weights), Fig. 3 (numbers of blast cells) and Table 1 (histology), it can be concluded that DNCB painting causes a strong cellular and humoral response. Histologically the size of the paracortical area at 4 and 6 days and the number of germinal centres at 10 days can be regarded as an indication of a development of CMI and a humoral response respectively (Turk and Oort, 1967). Sensitization by injection of DNP-erythrocytes and DNP-guinea-

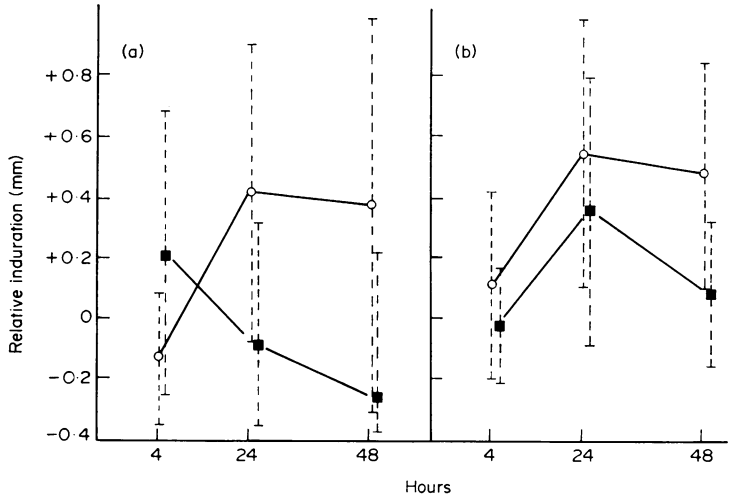


FIG. 7. Skin tests with DNP-erythrocytes, 10–12 days, after sensitization with (○) DNCB, and (■) DNP-erythrocytes. (a) Nine guinea-pigs per group (one test site per animal). (b) Seven guinea-pigs per group (two sites per animal). Relative indurations were calculated by subtracting at random the induration found in guinea-pigs injected with unconjugated erythrocytes from the induration in the test groups.

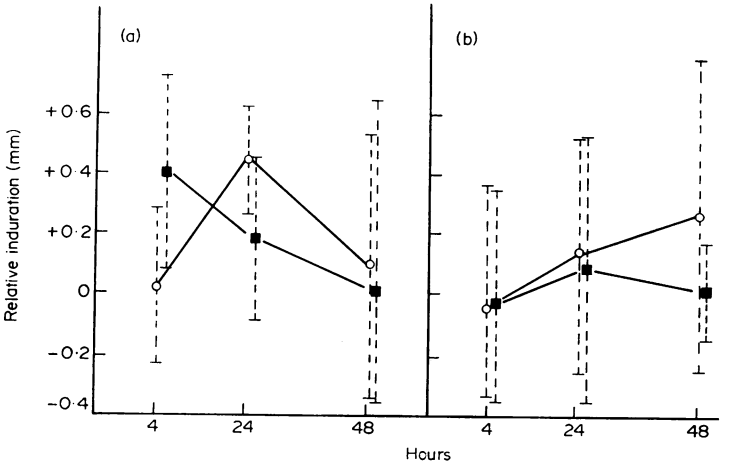


FIG. 8. Skin tests with DNP-erythrocyte-ghosts, 10–12 days after sensitization with (○) DNCB, and (■) DNP-erythrocytes. (a) Nine guinea-pigs per group (one test site per animal). (b) Seven guinea-pigs per group (one test site per animal).

pig serum in contrast shows no clear indication of a development of CMI or humoral immunity as judged by the same parameters.

The development of immunoblasts in lymphoid tissue also reflects the differences between the sensitization methods used.

In contrast, antibodies to DNP-erythrocytes, as assessed by haemagglutination, can be found after both DNCB sensitization and immunization with DNP-erythrocytes. The titre after sensitization by contact with DNCB tends to be lower than after sensitization by injection of DNP-erythrocytes. There are several explanations for this difference from the histological findings. One of these is the different carrier-specificity of antibodies, since the haemagglutination assay is carried out with only one of the possible hapten–carrier combinations, which are presumed to play a role in DNCB sensitization (Parker and Turk, 1970; Nakagawa and Tanioku, 1972).

The lower antibody titres found after injection of DNP-guinea-pig serum can be explained by the same arguments or by a lower antigenicity of the conjugate. These low titres, the slight histological reactivity and the fact that no RFC were found after injection with DNP-guinea-pig serum, made us decide to concentrate on the guinea-pigs sensitized with DNCB and those immunized with DNP-erythrocytes.

The results of skin testing these guinea-pigs for delayed type hypersensitivity were in accordance with the histological findings. Contact sensitivity to DNCB and delayed hypersensitivity to DNP-erythrocytes were strong in the DNCB-sensitized group, but were weak in the DNP-erythrocyte-injected group (Figs 7 and 8, Table 4). These results agree with the findings of Nakagawa and Tanioku (1972), who described such a reactivity after testing DNCB-painted guinea-pigs with DNP-conjugated epidermis extracts containing membrane fractions.

The numbers of DNP-erythrocyte RFC (Fig. 4) were compared with the data, obtained from guinea-pigs sensitized by painting with DNCB and those sensitized with DNP-erythrocytes. The higher number of RFC, found after DNCB sensitization, correlated with delayed skin reactivity as measured with DNP-erythrocytes, while no correlation was found with the humoral antibody titre, estimated with DNP-erythrocytes. These findings are in contrast to the findings of Roberts *et al.* (1971). They could not relate RFC to delayed hypersensitivity. Their relatively high background of RFC, attributable to their technique, might be of importance for an explanation of this difference.

Our histological data however do not support a definitive relationship between numbers of RFC and development of CMI. The lymph nodes of animals painted with DNCB contained relatively high numbers of RFC. Histological study of these nodes however demonstrated not only a strong paracortical enlargement, but also signs of a strong humoral response, as indicated by the number of germinal centres.

Apart from the problem of the correlation between numbers of RFC and CMI or humoral immunity, the problem of the specificity of these RFC was studied. The control experiments with DNCB and oxazolone-sensitized animals (Tables 2 and 3) do not support a complete specificity, since after sensitization by contact with DNCB not only an increase in the number of DNP-erythrocyte RFC but also a marked increase in the number of oxazolone-erythrocyte RFC and SRBC RFC was found. The higher number of these latter RFC can be explained on the basis of a larger number of antigen-reactive cells found in the unsensitized guinea-pigs. Several explanations for the non-specific formation of RFC can be given. One possibility is that this rosette formation is not an immunological phenomenon as suggested by Coombs, Gurner, Wilson, Holm and Lindgren

(1970) in connection with the human T-lymphocyte RFC. Such a hypothesis is not applicable to the DNP-erythrocyte RFC, because no higher number of these RFC was found after oxazolone painting, whereas oxazolone is known to be a strong sensitizer (Turk and Oort, 1967). Another explanation could be a cross-reactivity between the antigens used. Haritou and Argyris (1972) suggested that cross-reactivity could easily occur at the level of thymus-derived cells. This explanation is however unlikely in view of the antigens used in our experiments. The most probable explanation seems to us that the increase of the number of non-specific RFC is due to a non-specific proliferation of lymphoid cells in the lymph nodes. This may be caused by a release of lymphokines in the nodes (Kelly, Wolstencroft, Dumonde and Balfour, 1972).

In general our findings are not easy to interpret. They reflect the controversy in the literature as to the nature of RFC. We obtained data suggesting a relationship between numbers of RFC and CMI, whereas on the other hand our data suggest formation of RFC to be at least partly a non-specific phenomenon.

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