IMMUNOLOGICAL RESPONSES TO OXIDIZED FERREDOXIN AND ITS CHEMICALLY MODIFIED DERIVATIVES

by

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B.A. Luther College, 1971

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In the Department of

Microbiology

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

March, 1976

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ABSTRACT

Guinea pig lymph node and spleen cells responded in vitro to concanavalin A (con A), lipopolysaccharide (LPS), and a specific antigen, oxidized ferredoxin, in serum free medium, medium with mercaptoethanol (ME), medium with foetal calf serum (FCS), and medium with both FCS and ME. The addition of ME to serum free medium supported a mixed leucocyte reaction as did media with FCS or FCS and ME. Nylon wool fractionation of the cells eliminated the LPS response and treatment with anti-immunoglobulin (aIg) and complement (C') reduced the LPS response indicating that LPS may be a B lymphocyte mitogen. The con A response was enhanced by the aIg and C' treatment.

The specificities of the humoral and cellular immune responses to the amino terminal heptapeptide antigenic determinant of performic acid oxidized ferredoxin (O-Fd) were tested and compared using several synthetic peptides and analogues of the determinant in leucocyte migration inhibition tests with guinea pig spleen cells and inhibition of complement fixation between O-Fd and homologous rabbit antiserum. A tetrapeptide comprising the four amino acids at the carboxy terminal of the test determinant was able to produce significant inhibition of leucocyte migration. Modifications of amino acids within the tetrapeptide resulted in a loss of migration inhibition. This same peptide would only inhibit the complement fixation reaction if a hydrophobic group was attached to it. Otherwise a hexapeptide was required to produce significant inhibition of complement fixation. Guinea pigs sensitized with a conjugate of the heptapeptide determinant and bovine serum albumin gave positive immediate and delayed skin reactions to conjugates of the heptapeptide with other proteins indicating that the heptapeptide functions as both a hapten and carrier. Also, migration of spleen cells from these animals was inhibited by O-Fd.
The abilities of several chemically modified forms of ferredoxin to stimulate DNA synthesis in vitro in spleen cells from mice sensitized to O-Fd and to fix complement (C') in the presence of rabbit anti-O-Fd sera were tested. Of all the ferredoxins, only O-Fd, native ferredoxin (native-Fd) and N-ethylmaleimide alkylated ferredoxin (NEM-Fd) were able to stimulate DNA synthesis. O-Fd, native-Fd, acid precipitated ferredoxin (TCA-Fd) and dinitrophenylated O-Fd (DNP-O-Fd) fixed C' while methylated ferredoxin (meth-O-Fd) did not. NEM-Fd and carboxymethylated ferredoxin (CM-Fd) fixed C' weakly. Only native-Fd, O-Fd and NEM-Fd were found to be immunogenic in mice when assayed for lymphocyte stimulation by all of the ferredoxins. The 24 hour DNA synthetic response was sensitive to treatment with anti-mouse immunoglobulin and C' and anti-brain associated θ and C' while the 120 hour response was only sensitive to the anti-θ and C'.
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INTRODUCTION

The ability of the immune system to specifically recognize and react with antigens has been known for some time, but only recently has this been examined in detail using chemically defined, naturally occurring determinants. Previous work has often been done without knowledge of the location or nature of the determinants present on the antigen. As a result, most of these studies, which were done using antigens that were nonspecifically modified by a variety of methods, do not relate the specificities of the humoral and cellular immune responses to a single determinant. To an unknown degree the observed effect of such modifications may be due to alterations in the accessibility of the determinants by changes in the conformation of the molecule rather than modification of the determinants themselves. However, a number of interesting results have been found in several laboratories using undefined antigens and chemical modifications of them. Utilizing a variety of techniques to assay humoral and cellular immunity both in vivo and in vitro to chemically modified antigens, it has been frequently observed that the cellular immune response expresses a greater degree of cross reactivity than does the humoral response.

There have been several reports of work done with defined antigens, but in nearly all cases the antigens used for these studies were recognized by either thymus dependent lymphocytes (T cells) or antibodies. Direct comparisons of the specificity of T cells and antibody to the same determinant were not possible in those studies. It seems clear that testing the cross reactivity of T cells and antibody (or thymus independent B cells) to a single defined determinant is required to establish the relative specificities
of these responses.

A promising approach to the specificity problem was found when the antigenic nature of ferredoxin was elucidated. Ferredoxin is a 55 residue polypeptide isolated from *Clostridium pasteurianum*. It has been demonstrated that the performic acid oxidized form of ferredoxin (O-Fd) has only two major, detectable antigenic determinants to which antibodies are formed. Both of the determinants have been characterized and each is recognized by T cells and antibody, thus providing the opportunity for a direct comparison of specificities. In this thesis the specificity of the immune response to ferredoxin and its amino terminal determinant, N7, was tested using modified ferredoxins and synthetic analogues of the N7 determinant by assays for cell mediated immunity and serum antibodies.

The *in vitro* lymphocyte stimulation assay used in this study to examine specificity is generally believed to be a correlate of cell mediated immunity, although there has been some doubt raised recently of its validity as a measure of the cellular immune response. For this reason the parameters of the assay were extensively examined using several means of inducing DNA synthesis. Certain aspects of the assay commonly assumed to be necessary for a response, such as the requirement for foetal calf serum and long incubation periods prior to labelling with precursors to DNA, were tested. The types of cells (T or B) required to produce a proliferative response were also investigated. This work was begun using guinea pig cells and later continued using mouse cells to take advantage of the large volume of work already done that has characterized the immune system in mice making them more useful for immunological studies.
RESPONSES OF GUINEA PIG LYMPHOCYTES TO MITOGENS, AN ANTIGEN, AND MIXED LEUCOCYTE CULTURE IN MEDIA WITH AND WITHOUT MERCAPTOETHANOL AND FOETAL CALF SERUM

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SUMMARY

The ability of guinea pig spleen and lymph node cells to undergo a proliferative response in vitro in the presence of mitogens (concanavalin A and lipopolysaccharide), a specific antigen (oxidized ferredoxin), and allogeneic cells was assessed under a variety of conditions. Time and dose dependency of the responses was measured in RPMI 1640 (1640), 1640 plus mercaptoethanol (ME), 1640 plus foetal calf serum (FCS), and 1640 with ME and FCS. Mitogen responses were also measured after treatment of the cells with sheep anti-guinea pig immunoglobulin (SaGPIg) and complement (C') or after passage through nylon wool columns. Lipopolysaccharide (LPS) stimulated the cells under all media conditions over a wide range of concentrations but over a narrow time period. Nylon wool treatment of the cells eliminated the LPS response while SaGPIg and C' reduced it. Concanavalin A (con A) stimulated the cells under all test conditions and demonstrated a dose-time interrelationship in terms of maximum response. Pre-treatment of cells with SaGPIg and C' enhanced the response to con A while nylon wool fractionation diminished it somewhat. Only lymph node cells responded in vitro to oxidized ferredoxin (O-Fd). In serum free media the O-Fd responses were maximal at 48 hours whereas in media containing FCS proliferative responses were supported for a prolonged period and appeared to be bimodal. Except for an early response with 1640 and ME, only media containing FCS supported stimulation in the mixed leucocyte culture (MLC).
INTRODUCTION

The in vitro stimulation of lymphocytes by mitogens, antigens or allogeneic lymphoid cells has been used as a correlate of cell mediated immunity except in instances where B lymphocyte mitogens were used. However, there is some question regarding what populations of cells are responding and whether or not macrophages are involved (Mugraby, Gery, and Sulitzeanu, 1974). Various types of responses of lymphoid cells to a number of mitogens have been demonstrated in several animal species. In the mouse it is recognized that different lymphoid cell populations will respond to certain mitogens. Con A and LPS have been shown to be specific mitogens for T and B lymphocytes, respectively (Anderson, Möller, and Sjöberg, 1972b), although it has been demonstrated that con A will stimulate B lymphocytes if it is appropriately presented (Anderson, Edelman, Möller, and Sjöberg, 1972a).

Murine T lymphocytes may be separated by passage of lymphocytes through nylon wool columns (Julius, Simpson, and Herzenberg, 1973), or by treatment with anti-mouse immunoglobulin plus C' (Takahashi, Old, McIntire, and Boyse, 1971). Conversely, populations of cells free of T lymphocytes may be prepared by treatment with anti-Θ and C' (Raff, 1969; Lamelin, Lisowska-Bernstein, Matter, Ryser, and Vassalli, 1972). The guinea pig system has not been so well elucidated. In the present study, treatment of cells with nylon wool or SaGPIg and C' was examined to observe their effects on the con A and LPS responses.

Recently, reports have appeared demonstrating that lymphocytes can
be cultured for relatively short periods of time without serum (Vischer, 1972; Coutinho, Möller, Andersson, and Bullock, 1973). *In vitro* systems in which serum can be eliminated have several advantages; lower background controls, simpler characterization of supernatant factors, elimination of variability between batches of serum, and reduction of non-specific stimulation.

Several investigators have reported that the addition of reducing agents such as mercaptoethanol and cysteine has a beneficial effect in their culture systems. Greater viability and enhanced response to antigens, mitogens, and MLC have been claimed (Chen and Hirsch, 1972; Bevan, Epstein, and Cohn, 1974; Broome and Jeng, 1973; Fanger, Hart, Wells and Nisonoff, 1970; Heber-Katz and Click, 1972).

This study was undertaken to elucidate some of the parameters governing the proliferative response of guinea pig lymph node and spleen cells to various stimuli when cultured in media with and without FCS and/or ME, and to correlate some of the data presented here with information already available in murine systems.
MATERIALS AND METHODS

ANIMALS

Outbred albino guinea pigs of either sex weighing approximately 400 grams were used in all experiments.

MITOGENS

Con A (Sigma, St. Louis, Mo.) was made up in phosphate buffered saline (PBS) to 200 μg/ml, sterilized, and stored frozen. LPS-W from S. typhimurium (Difco, Detroit, Mich.) was dissolved in PBS at 10 mg/ml, the pH adjusted to 8.0, heated for 30 minutes in a boiling water bath and stored frozen. All further dilutions were made in serum free medium.

ANTIGEN PREPARATION AND IMMUNIZATION

Ferredoxin from C. pasteurianum (Sigma) was performic acid oxidized (Mitchell, Levy, and Nitz, 1970) before use. Animals were injected in 5 locations with 250 μg of O-Fd in 50% complete Freund's adjuvant (CFA) using a total volume of 0.5 ml per animal. The animals were boosted similarly after 14 days and sacrificed 10 days later. O-Fd used in cultures was dissolved in medium, sterilized, and stored frozen.

PREPARATION OF CELLS

Spleens and lymph nodes were removed aseptically, teased into PBS and the resulting cell suspensions transferred to plastic tubes. The cells were spun down (180 x g for 5 min.) and resuspended in 0.85% NH₄Cl (in 0.01 M PO₄, pH 7.2) for 4 to 5 minutes to lyse the red blood cells. After 3 washes with PBS they were counted using trypan blue to assess viability.
MEDIA

The basic medium used for all experiments was RPMI 1640 (Gibco, Grand Island, New York) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml fungizone. Mercaptoethanol was made up aseptically in medium at $2.5 \times 10^{-4}$ M and stored frozen. Foetal calf serum (Gibco, #84557) was inactivated at 56°C for 30 min. and made up to 20% in medium.

CELL CULTURE

Spleen and lymph node cells were cultured in microculture plates. Mitogens or antigen in medium were added in a volume of 0.05 ml/well. When ME or FCS was used, 0.05 ml of the previously described stock solutions was added to each well. Five to $10 \times 10^5$ cells in 0.10 ml of medium were added to each well along with whatever volume of medium was required to make a final volume of 0.25 ml. This procedure resulted in a final concentration of ME at $5 \times 10^{-5}$ M, FCS at 4%, and cells between 2-4 $\times 10^6$/ml. The MLC's were set up in a similar manner except that $2.5 \times 10^5$ cells in 0.05 ml from each animal were used per well. Control wells contained 5 $\times 10^5$ unmixed cells. Background counts were obtained by averaging the control counts of each animal.

LABELLING AND HARVESTING

Tritiated thymidine (Amersham-Searle, Arlington Heights, Ill. specific activity 2.0 Ci/m mole) at a concentration of 1.0 μCi in 0.05 ml was added to the wells 18 hours before harvesting. Harvesting was performed by aspirating the contents of each well onto glass fiber filters using a
multiple sample harvestor (Hartzman, Bach, Bach, Thurman, and Sell, 1972; Thurman, Strong, Ahmed, Green, Sell, Hartzman, and Bach, 1973). The filters were dried and counted in a scintillation counter.

NYLON WOOL TREATMENT

Cells treated with NH₄Cl and washed as described above were suspended in PBS plus 5% FCS and placed in plastic tissue culture plates for 1 hour at 37°C to remove adherent cells. PBS with 5% FCS was used for all subsequent nylon wool column manipulations. The non-adherent cells were washed into a nylon wool column, incubated for 1 hour at 37°C and eluted slowly. The column consisted of a 25 ml syringe barrel filled to the 20 ml level with loosely packed nylon wool and sterilized. The procedure was adapted from that used by Julius et al. (1973). Recovery of cells was approximately 15 to 20% of those applied. To obtain sufficient numbers of cells for some experiments, lymph node and spleen cells were pooled before filtration. These cells are referred to as pool cells.

KILLING WITH ANTI-Ig PLUS COMPLEMENT

Ammonium chloride treated cells were taken up in sterile, inactivated SaGPIg or guinea pig C' (each was diluted 1:4 in PBS) or in both and incubated with intermittent mixing for 1 hour at 37°C after which the cells were washed and counted. 0.2 ml of the diluted SaGPIg and C' were used per 10⁷ cells. Where necessary, lymph node and spleen cells were pooled before treatment to ensure adequate numbers of cells.
RESULTS

INDUCTION OF DNA SYNTHESIS BY LPS

The results of dose response and kinetics experiments using spleen cells in serum free medium are presented in Fig. 1A. The optimal dose was found to be 4 μg/ml after 24 hours in culture. In cultures with FCS, concentrations of 16 and 64 μg/ml stimulated well after 24 hours (Fig. 1B). Stimulation indices (SI) between 2.2 and 3.1 were found with LPS induced spleen cells in medium plus ME at LPS concentrations from 4-256 μg/ml after 24 hours in culture. LPS stimulated lymph node cells as shown in Table 1. The dose response to LPS was quite broad in all tests carried out. Pooled cells did not respond as well as spleen or lymph node cells alone (Table 1).

INDUCTION OF DNA SYNTHESIS BY CON A

Similar experiments were done using con A. The results of spleen cell stimulation in serum free medium are presented in Fig. 2A. The maximum response was found after 48 hours of culture using 1 μg/ml. Higher doses (4 and 8 μg/ml) induced moderate transformation after only 24 hours. The response with respect to the SI in medium with FCS was lower than the serum free response (Fig. 2B). With FCS, peak stimulations were induced by 32 μg/ml at 24 hours and 1 μg/ml at 48 hours of culture. In con A experiments there was a trend toward high doses stimulating early and lower doses peaking at a later time, usually 48 or 72 hours. Lymph node cells produced much greater activation to con A than did spleen cells.
Typical stimulations with 2 µg/ml con A over 24, 48, and 72 hours were between 40 and 60 fold in all media tested. As observed with the LPS results, the highest SI's were found using serum free media.

**EFFECT OF ANTI-Ig AND C' OR NYLON WOOL ON THE RESPONSE TO LPS AND CON A**

In most cases pretreatment of the cells with SaGPIg and C' enhanced the con A response and moderately reduced the response to LPS (Table 1). Only treatment of the cells with nylon wool consistently eliminated the LPS response; however, the con A response was also diminished somewhat (Table 1). Treatment with SaGPIg and C' killed 23% of spleen cells and 20% of lymph node cells compared to controls containing normal sheep serum or C' alone or SaGPIg alone.

**ANTIGEN-INDUCED LYMPHOCYTE TRANSFORMATION**

The responses to O-Fd found in serum free medium (Fig. 3A) and medium plus ME (Fig. 3B) were very similar. In both cases the response peaked at 48 hours with 32 µg/ml in medium only and 16 µg/ml in medium with ME. By 72 hours the SI had dropped sharply and by 96 and 120 hours was below unstimulated controls. The responses in medium plus FCS (Fig. 3C) and medium with FCS and ME (Fig. 3D) were bimodal. Both media supported good responses at 24 hours which declined at 48 and 72 hours and peaked again at 96 and 120 hours. The concentrations of O-Fd which induced the later response were often lower than those which stimulated the 24 hour response. Spleen cells did not respond under any of the conditions used even though lymph node cells from the same animal were able to respond. Lymph node and
spleen cells from control animals immunized with CFA only were not
stimulated by O-Fd.

MLC RESPONSE OF LYMPH NODE AND SPLEEN CELLS

Data from the MLC tests has been summarized in Table 2. Medium
alone was unable to support a response at anytime during the culture
period. Except in the case of lymph node cells at 96 hours, very little
stimulation was found in medium with ME. Media with FCS or FCS and ME
supported normal responses, however, the response with FCS and ME was
better than in medium with FCS only.
DISCUSSION

The results presented here have shown that guinea pig lymphocytes can be readily cultured and stimulated by mitogens and an antigen (O-Fd) in serum free medium. With the addition of ME and/or FCS to the medium, a mixed lymphocyte reaction was also detected.

Media composition had a marked effect on the control background counts in the stimulations. Average background counts per minute when cultures were tritiated at 24 hours were 899 (medium only), 2210 (medium plus ME), 1825 (medium and FCS), and 7252 (medium with FCS and ME). Although actual counts in mitogen stimulations were higher in media containing ME and/or FCS, the stimulation indices were, on the average, higher in media without serum and ME because the unstimulated controls were lower. The SI's of O-Fd cultures were similar regardless of the type of media used, while the MLC's were consistently better when the medium was supplemented with FCS and ME.

A wide range of LPS concentrations (0.25-256 μg/ml) was able to induce DNA synthesis and in the presence of ME there was little difference in the response of cells to 4-256 μg/ml LPS. The kinetics of the LPS response was unusual in that, regardless of the media used, significant stimulation was achieved only when the cultures were tritiated at 24 hours. At 0 and 48 hours only slight stimulation was found and by 72 and 96 hours the counts were below the unstimulated controls. The addition of FCS or ME shifted the dose response to slightly higher LPS concentrations.

Both the dose response and kinetics of the con A stimulations were moderately altered by the use of FCS. Except for con A at 1 μg/ml, cultures
containing FCS usually required higher concentrations of con A and the response was more prolonged. Other workers have noted that higher concentrations of con A are required in media with FCS and attribute this to the ability of con A to bind serum proteins (Möller, Andersson, Pohlit, and Sjöberg, 1973).

Con A–induced DNA synthesis was substantially higher with respect to total counts and SI with lymph node cells than with spleen cells. This observation is consistent with the generally accepted view that there are greater relative numbers of T lymphocytes in lymph nodes than in spleens. It has been proposed that con A is a T lymphocyte specific mitogen in chickens (Weber, 1973), guinea pigs and rabbits (Elfenbein, Harrison, and Green, 1973), and mice (Elfenbein et al., 1973; Andersson et al., 1972b).

The results of our experiments using nylon wool and anti-Ig with C' are in agreement with studies in other animal systems which indicate that LPS is a B lymphocyte mitogen. Using different techniques, Elfenbein et al. (1973) have suggested that LPS is B lymphocyte specific in guinea pigs. Using anti-light chain and C', Gmelig Meyling, Kooy-Blok, and Ballieux (1974) were able to kill 12% of peripheral blood lymphocytes and 42% of lymphocytes from human tonsils. Takahashi et al. (1971) using anti-light chain and C' in mice were able to kill up to 50% of spleen lymphocytes and 40% of lymph node lymphocytes. Our results of 23% killing of spleen cells and 20% killing of lymph node cells are consistent with these findings since spleen and lymph node cells rather than enriched populations of lymphocytes were used. The ability of nylon wool columns to remove cells bearing surface Ig (presumably B cells) has been demonstrated by Julius et al. (1973).

In our experiments, nylon wool treatment of guinea pig cells completely eliminated the response to LPS, whereas treatment with anti-Ig and C' only reduced it. Using rabbit-anti mouse immunoglobulin and C' we have
been able to reduce the LPS response of mice by more than 80% (unpublished observations). That the guinea pig LPS response is relatively insensitive to SaGPIg and C' treatment could be due to an LPS responsive population of cells with a low density of surface immunoglobulin. Nylon wool treatment also reduced the con A response. As nylon wool operates on a principle of differential adherence, it is possible that some of the cells capable of responding to con A are removed. Usually, the con A response was enhanced by treatment with anti-Ig and C'.

O-Fd induced thymidine uptake in lymph node cells only, but in all of the media tested. Vischer (1972) using mice and Kirchner and Oppenheim (1972) with chickens have reported serum free antigen stimulation using KLH and sheep red blood cells, respectively, as antigens. The kinetics of the serum free O-Fd responses with and without ME were similar to each other and distinctly different from the kinetics of medium with FCS and medium with FCS and ME. It was unexpected to see the antigen stimulation in FCS and FCS plus ME containing media occur as early as 24 hours, decline at 72 hours (a time often considered to be optimal) and increase again at 96 and 120 hours. These results may indicate that two different cell populations were responding to the antigen at different times. The bimodal response was not observed in serum free media, probably because without serum, cell viability was not adequate to support a second response. Preliminary experiments, not presented here, testing the response of anti-Ig and C' or nylon wool treated cells to O-Fd have been inconclusive but suggest that SaGPIg and C' treated cells may be able to respond, whereas nylon wool treated cells seem incapable of responding. Mugraby et al. (1974)
using mice demonstrated that depletion of T or B lymphocytes reduced the response to sheep red blood cells.

The use of ME had its most prominent effect in the MLC tests. The response in medium plus FCS and ME was greater and more prolonged than in medium with FCS only. No response was found in serum free medium, but with the addition of ME, a detectable response was obtained. Bevan et al. (1974) using mouse spleen cells showed that addition of ME to serum free MLC tests resulted in positive responses. Similar results were obtained by Heber-Katz and Click (1972). Although the mechanism of action of ME and related compounds is unknown at this time, it has been observed that the presence of ME, cysteine, etc., greatly increases the viability of cells in culture (Chen and Hirsch, 1972; Heber-Katz and Click, 1972). Chen and Hirsch (1972) have used ME as a substitute for macrophages which also appear to have a beneficial effect on viability. It has been proposed that ME acts as a substitute for a macrophage produced factor (Broome and Jeng, 1973).

It would appear that those responses which require prolonged culturing of lymphocytes cannot be supported in media lacking FCS, as noted in the late stimulation by O-Fd, or ME, as seen in the MLC tests. However, responses which are maximal within the early days of culture are adequately supported in the absence of these media components. The data reported here indicate that there are no major differences between the responses of guinea pig and mouse lymphocytes in the mitogen, antigen, and MLC test systems.
TABLE 1

Effect of Nylon Wool or SaGPIg and C' Treatment on the Con A and LPS
Responses of Guinea Pig Cells

<table>
<thead>
<tr>
<th>Medium supplements</th>
<th>Source of culture</th>
<th>Hours of labelling</th>
<th>Treatment</th>
<th>Con A µg/ml</th>
<th>LPS µg/ml</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
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<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>nylon wool</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FCS and ME</td>
<td>spleen</td>
<td>24</td>
<td>none</td>
<td>10.5*</td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>anti-Ig and C'</td>
<td>9.10</td>
<td>5.02</td>
</tr>
<tr>
<td>FCS and ME</td>
<td>node</td>
<td>24</td>
<td>none</td>
<td>42.9</td>
<td>33.2</td>
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<tr>
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<td></td>
<td></td>
<td>anti-Ig and C'</td>
<td>70.7</td>
<td>50.8</td>
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<tr>
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<td>pool</td>
<td>24</td>
<td>none</td>
<td>27.4</td>
<td>-</td>
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<td></td>
<td></td>
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<td>nylon wool</td>
<td>14.6</td>
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<td></td>
<td></td>
<td>anti-Ig and C'</td>
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<td>-</td>
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<tr>
<td>FCS and ME</td>
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<td>48</td>
<td>none</td>
<td>-</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>nylon wool</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Stimulation Index
TABLE 2

MLC Tests of Allogeneic Guinea Pig Lymph Node and Spleen Cells
in FCS and ME Supplemented Media

<table>
<thead>
<tr>
<th></th>
<th>Medium Only</th>
<th>Medium Plus ME</th>
<th>Medium Plus FCS</th>
<th>Medium Plus ME and FCS</th>
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</tr>
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<td>1.08</td>
<td>1.33</td>
<td>1.08</td>
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<tr>
<td></td>
<td>0.93</td>
<td>0.92</td>
<td>2.00</td>
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<td></td>
<td>0.89</td>
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<td></td>
<td>0.96</td>
<td>1.20</td>
<td>0.76</td>
<td>1.28</td>
</tr>
<tr>
<td></td>
<td>1.22</td>
<td>1.34</td>
<td>0.76</td>
<td>1.44</td>
</tr>
</tbody>
</table>

** Source of cells

* Stimulation Index

# The cultures were labeled for 18 hours after incubation for the indicated length of time
Fig. 1. The response of guinea pig spleen cells to LPS in (A) serum free medium and (B) medium with 4% FCS. The cultures were labelled with tritiated thymidine for 18 hours after the indicated hours in cultures 0, 0—0; 24, □—□; 48, △—△; 72, •—•; and 96, ■—■.
Fig. 2. The Con A responses of guinea pig spleen cells in (A) serum free medium and (B) medium with 4% FCS. An 18 hour labeling with tritiated thymidine was done after 0, 0---0; 24, □---□; 48, △---△; 72, ○---○; 96, ■---■; and 120, ▲---▲; hours in culture.
Figure 2A
Fig. 3. The response of O-Fd immune guinea pig lymph node cells to O-Fd at 1, O--O; 2, □-□; 4, Δ--Δ; 8, ●-●; 16, ■-■; and 32, ▲-▲; μg/ml. The cultures were labeled for 18 hours beginning at the times indicated on the figures. The media used were (A) medium only, (B) medium with ME, (C) medium with FCS, and (D) medium with FCS and ME.
REFERENCES


THE IMMUNE RESPONSE TO FERREDOXIN

I. SPECIFICITY OF THE RESPONSE TO THE AMINO TERMINAL DETERMINANT

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SUMMARY

Several synthetic peptides and analogues of the amino terminal antigenic determinant of oxidized ferredoxin (O-Fd) were tested for their ability to inhibit the complement fixation reaction between O-Fd and homologous antiserum, and to inhibit the migration of spleen cells from animals immunized to O-Fd or to a conjugate of its amino terminal heptapeptide \( (N_7) \) and bovine serum albumin \( (N_7\text{-BSA}) \). The heptapeptide \( (H_2N\text{-ala-tyr-lys-ile-ala-asp-ser-COOH}) \) is known to be an antigenic determinant of O-Fd. The results of the migration inhibition assay suggest that the carboxy terminal tripeptide of the heptapeptide is partially able to stimulate the production of migration inhibition factor. The tetrapeptide and longer peptides of the native sequence were all recognized to a much greater degree than the tripeptide. Peptides modified at the aspartic residue were partially active while the serine modified peptide was not. Modification at the amino end of the heptapeptide had no effect on migration inhibition. A control peptide containing the same amino acids in an altered sequence was not recognized. The peptides were tested with unimmunized spleen cells to eliminate the possibility of cytotoxicity or nonspecific inhibition of migration. As additional specificity controls, it was shown that the \( N_7 \) peptide and the \( N_7\text{-BSA} \) conjugate inhibited migration in O-Fd immunized animals, while O-Fd inhibited migration in \( N_7\text{-BSA} \) immunized animals.

The hexa, hepta, aspartic deleted and serine modified peptides were able to inhibit the complement fixation reaction with O-Fd and specific rabbit antiserum. Inhibition found with the serine modified peptide and the lack of inhibition with the amino modified peptide or the di, tri, and...
tetrapeptides indicates that the determinant recognized by the rabbit antibodies is either larger than the determinant which induces the production of MIF or is located nearer to the middle of the heptapeptide. The control peptide did not inhibit complement fixation.
INTRODUCTION

The specificity of antigen recognition by antibodies and thymus derived lymphocytes (T cells) has been examined in the past. Several of these studies have used chemically modified antigens with uncharacterized determinants. Parish (1972) using several heterologous red blood cells and chemical modifications of them demonstrated significant cross reactivity at the T cell level and an inverse correlation in the abilities of the various modified and unmodified red blood cells to stimulate delayed hypersensitivity or antibody production. Similar results were found previously using native and acetoacetylated flagellin (Parish, 1971 a,b). Using modified albumins, Schirrmacher and Wigzell (1972) and Rubin and Wigzell (1973) also demonstrated a greater degree of cross reactivity in the cell mediated immune response than in the antibody response. Hoffman and Kappler (1973) using several heterologous red blood cells showed that cross reaction at the T cell level was greater than that seen with antibodies. There are a number of other reports which imply that the cellular immune response has a wider cross reactivity than the antibody response (Maron, Webb, Teitelbaum and Arnon, 1972; Schirrmacher and Wigzell, 1974; Coon and Hunter, 1973; Dailey and Hunter, 1974; Champlin and Hunter, 1975). These studies have been limited by the lack of defined determinants to which both T cells and antibody are directed, making comparisons of specificity questionable.

The use of hapten-carrier systems (DNP-protein conjugates, etc.) has in most cases produced predominantly antihapten antibodies and anti-carrier
cell mediated immunity (Katz, Paul, Goidl and Benacerraf, 1970; Paul, Katz, Goidl and Benacerraf, 1970; Davie and Paul, 1971) eliminating the ability to compare specificities. In those cases where anti-DNP cell mediated immunity has been produced, the specificity of the responses has not been compared (Janeway, Cohen, Ben-Sasson and Paul, 1975; Goscicka, 1974).

The antigenic specificity of T cells has also been tested using small immunogenic molecules, especially azobenzene arsonate-L-tyrosine (ABA-L-tyr). Data from several laboratories has indicated that certain minor modifications to the amino and carboxy groups of the tyrosine moiety or to the arsonate group do not result in a complete loss of recognition. As no antibody is produced to ABA-L-tyr, these experiments do not permit a direct comparison of antibody and T cell recognition (Hanna and Leskowitz, 1973; Jokipii and Jokipii, 1974; Becker, Levin and Sela, 1973; Alkan, Williams, Nitecki and Goodman, 1972; Bush, Alkan, Nitecki and Goodman, 1972).

More definitive work has been done on the coat protein of tobacco mosaic virus demonstrating that the minimum size of a determinant recognized by antibodies is 5 amino acid residues. These small peptides were found to be nonimmunogenic and did not stimulate DNA synthesis in vitro with sensitized lymphoid cells, but did give positive delayed skin reactions and stimulated the production of migration inhibition factor (MIF). Conservatively modified peptides were recognized and a difference in the specificity of recognition was noted between anti-sera from different rabbits (Benjamini, Shimizu, Young and Leung, 1969; Spitler, Benjamini, Young, Kaplan and Fudenberg, 1969).
Specificity studies done on glucagon, a small polypeptide of 29 amino acid residues, have indicated the presence of two antigenic determinants, one in each half of the molecule. The amino terminal determinant is strongly recognized by anti-glucagon antibodies and also stimulates MIF production. The carboxy terminal determinant has less antibody directed against it, but stimulates DNA synthesis and MIF production in vitro (Senyk, Williams, Nitecki and Goodman, 1971). Thus, the amino terminal determinant has greater "hapten" activity while the carboxy terminal determinant has greater "carrier" activity although neither is exclusively antibody or T cell directed.

Work in this laboratory with ferredoxin isolated from Clostridium pasteurianum has revealed that this polypeptide of 55 amino acid residues has only two major detectable determinants, the amino terminal heptapeptide \( N_7 \) and the carboxy terminal pentapeptide \( C_5 \) (Nitz, Mitchell, Gerwing and Christenson, 1969; Mitchell, Levy and Nitz, 1970; Mitchell and Levy, 1970; Kelly and Levy, 1971). Both antibodies and T cells recognize each of these determinants with \( N_7 \) being slightly more T cell directed and \( C_5 \) stimulating the production of a somewhat greater quantity of antibody (Kelly and Levy, 1971; Waterfield, Levy, Kilburn, and Teather, 1972; Levy, Hull, Kelly, Kilburn and Teather, 1972; Pearson, Levy and Kilburn, 1975). A considerable amount of work has been done with the \( N_7 \) determinant since it is readily synthesized, contains several modifiable amino acids and can be labeled to a high specific activity with \( ^{125}I \) or \( ^3H \) labeled acetic anhydride. Neither of these labeling procedures causes a loss of recognition (Kelly and Levy, 1971; Pearson et al, 1975).
In this study, the fine specificity of the immune response to the N\textsubscript{7} determinant has been examined by using several peptide analogues of N\textsubscript{7}. Inhibition of the complement fixation reaction has been used to assay antibody specificity. The leucocyte migration inhibition test, classically associated with delayed hypersensitivity and cellular immunity (Salvin and Smith, 1960; David, Al-Askari, Lawrence and Thomas, 1964; David, Lawrence and Thomas, 1964; David and Schlossman, 1968) was used to determine T cell specificity. The results described here compare the specificity of recognition by antibodies and T cells to a single determinant.
MATERIALS AND METHODS

ANIMALS

Young adult outbred albino rabbits and guinea pigs were used for all experiments.

PEPTIDES

Several peptides of various structures were prepared by solid phase peptide synthesis as described by Hancock, Prescott, Marshall and Vagelos (1972), a modification of the Merrifield synthesis (Merrifield, 1963). The peptides and nomenclature are shown in Table 1. The GEE-N7 peptide was made by reacting the N7 peptide with 20 equivalents of glycine ethylester and 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDCI) at pH 4.7 for 4 hours (Hoare and Koshland, 1967). The N4-Bzl-Ser peptide was synthesized by solid phase except that it was cleaved from the resin by transesterification with dimethylaminoethanol. The resulting esters were removed by hydrolysis in DMF-water (1:2) leaving the O-benzyl ether blocking group intact on the serine hydroxyl. The procedure is that published by Savoie and Barton (1974). The N-M-Asp peptide was an error peptide isolated from the N7 synthesis. DNP-N7 was a gift from D. Waterfield. The peptides were routinely purified by Sephadex G-15 gel filtration in 0.05 M acetic acid and ion exchange on Dowex 1 x 2 resin using pyridine-acetic acid-water buffers. Peptide purity was assessed by thin layer chromatography on silica gel G using at least 3 solvent systems. Further tests of purity and quantitation were performed on a Beckman 120 amino acid analyzer. Molar ratios from the amino acid analyses are shown in Table 2.
ANTIGENS

Ferredoxin from *C. pasteurianum* was purchased from Sigma (St. Louis, Missouri) or isolated from cultures as described by Mortenson (1964) and Tanaka, Nakashima, Mower and Yasunobu (1964). Oxidation was performed by dissolving the ferredoxin (20-40mg) in 1.66 ml formic acid followed by 3.33 ml of performic acid (3.0 ml of formic acid plus 0.33 ml of 30% hydrogen peroxide). The reaction was allowed to continue for 2 hours at -10°, after which the reaction mixture was evaporated under vacuum, dialyzed, concentrated and analyzed for amino acid content and quantity. The N\textsubscript{7}-BSA conjugate was made by mixing N\textsubscript{7} and BSA (20:1) in the presence of EDCI according to Hoare and Koshland (1967). After 4 hours reaction the solution was dialyzed, concentrated and analyzed to determine the degree of coupling. N\textsubscript{7} was also conjugated to poly-D-glutamic acid (PDG, MW-75,000 Sigma) and poly-L-lysine (PLL, MW-17,000 Schwarz-Mann) by the same procedure. Substitution ratios as calculated from amino acid analyses before and after substitution were: N\textsubscript{7}-BSA, 6:1; N\textsubscript{7}-PDG, 10.3:1; N\textsubscript{7}-PLL, 12.6:1.

IMMUNIZATIONS

Rabbits were immunized intramuscularly in each leg several times at 2 week intervals with 1 mg doses of O-Fd in 50% Freund's complete adjuvant (FCA) to produce the antisera. Guinea pigs were immunized twice intramuscularly with either 250 μg of O-Fd or N\textsubscript{7}-BSA in 50% FCA.

SKIN TESTS

The shaved and depillated flanks of guinea pigs were injected intradermally with 50 μg of antigen in 0.1 ml saline. Controls consisted of
tests with 0.1 ml saline on immunized animals and complete tests with all antigens on unimmunized animals.

**LEUCOCYTE MIGRATION INHIBITION TEST**

Spleen cells from guinea pigs were washed twice in phosphate buffered saline (pH 7.2, 0.01 M PO₄) and resuspended in RPMI 1640 medium plus 5% heat-inactivated foetal calf serum at a concentration of 10% cells/media. The cells were drawn into 1 x 75 mm capillary tubes which were sealed with plastic putty (Fisher) and the cells sedimented at 200 x g for 5 minutes. The tubes were broken at the cell-medium interface and placed into chambers containing 1 ml of medium. Test peptides were used at a concentration of 0.05 μmoles/ml in the same medium as good inhibition was obtained at this concentration without cytotoxic effects. N₇-PDG, N₇-PLL and N₇-BSA were used at 10 μg/ml and O-Fd at 16 μg/ml. After 18 hours incubation in a 37°C, 5% CO₂ incubator, the areas of migration were measured using the calibrated stage of a microscope. Control experiments were performed in an identical manner on unimmunized animals. The procedure is similar to that described previously (Waterfield, et al, 1972; Levy, et al, 1972; Waterfield, Levy and Kilburn, 1974). The results are expressed as the ratio of the migration area found with a test antigen to the migration area of controls containing only medium. An inhibition was considered significant if the ratio of areas was less than 0.80 and the t-probability was less than 0.05.

**INHIBITION OF COMPLEMENT FIXATION**

The complement fixation tests were performed as described previously (Gerwing and Thompson, 1968) using rabbit anti-O-Fd antiserum which had been heat inactivated at 56°C for 30 minutes.
RESULTS

The ability of preformed antibody to O-Fd to recognize modified or partial N7 peptides was assessed by measurement of their ability to inhibit fixation of complement in the presence of O-Fd and the antibody at optimal proportions. The results are shown in Figure 1 A and B and represent the average of 4 individual experiments. Percent inhibitions were estimated at the range in which control tubes containing only O-Fd and antiserum yielded 50% hemolysis in the presence of complement. Of the partial N7 peptides, only N6 was capable of consistently inhibiting the reaction. This suggests that the portion of the N7 determinant recognized by antibody encompasses 6 of the 7 residues or is located centrally or near the amino terminal end. The possibility that the specificity of the recognition may involve amino acid residues at the amino terminal portion of the molecule is supported by the observation that DNP-N7 (Fig. 1B) was incapable of inhibiting the complement fixation reaction. Unlike observations made with the leucocyte migration inhibition test (see below), the peptide modified at the serine residue (N4-Bzl-Ser) and containing only the N4 amino acids was consistently able to inhibit complement fixation although N4 alone was unable to do so. It is possible that the O-Benzyl group may provide a hydrophobic enhancing effect as noted by others (Benjamini et al, 1969) without interfering with specificity. Unlike the migration inhibition tests, the peptide lacking aspartic acid (N-M-Asp) was also capable of inhibiting complement fixation, thus reinforcing the possibility that the specificity for antibody recognition lies centrally within the N7 determinant. Specificity of the reaction was established by negative results obtained with
NC\textsubscript{7} (the synthetic peptide containing the same amino acids as N\textsubscript{7}, but in a different sequence).

The specificity requirements for cellular immunity as assessed by the inhibition of leucocyte migration were measured using spleen cells from guinea pigs sensitized to O-Fd. Spleen cells were set up with peptides of various length, each constituting part of the N\textsubscript{7} determinant and with chemically modified analogues. Tests were also carried out with N\textsubscript{8-N} (a synthetic peptide of 22 amino acids containing two N\textsubscript{7} determinants linked by 8 glycine residues (see Kelly, Levy and Hull, 1973), O-Fd, N\textsubscript{7}-BSA, N\textsubscript{7}-PDG, N\textsubscript{7}-PLL and NC\textsubscript{7}. The results are summarized in Table 3. It can be seen that, in terms of size, the N\textsubscript{4} peptide was the smallest part of the N\textsubscript{7} peptide which consistently inhibited migration. Although it is not shown by the averaged data, it was observed that, occasionally, the N\textsubscript{3} peptide also caused inhibition; this was observed with spleen cells which demonstrated stronger than average inhibition in the presence of N\textsubscript{4} through N\textsubscript{7}. N\textsubscript{2} was consistently unable to inhibit migration. The NC\textsubscript{7} peptide had no effect on the migration thus establishing the dependence of the response on amino acid sequence rather than on overall charge effects. Tests run on peptides in which either the aspartic or serine residues had been modified showed minimal or no ability to inhibit leucocyte migration, implying the importance of the integrity of this end of N\textsubscript{7} in cellular recognition. All of the N\textsubscript{7} conjugates (N\textsubscript{7}-PDG, etc) were able to inhibit migration, emphasizing the lack of carrier effect. Both the data on the peptides of varying length (N\textsubscript{2}-N\textsubscript{7}) and the data on the serine and aspartic modified peptides indicate that the actual sequence recognized in both length and
configurational specificity is contained within the \( N_4 \) peptide, since longer peptides do not show a greater ability to inhibit migration, and peptides modified at the amino terminal or lysine residue (DNP-\( N_7 \)) are still specifically recognized by sensitized cells.

In the experiments described above, the immunogen in all instances was O-Fd. Further studies were carried out to determine the effect on the cell mediated response to the \( N_7 \) determinant when the \( N_7 \) peptide was conjugated with EDTA to BSA. Guinea pigs so immunized were subsequently tested for skin reactivity to the \( N_7 \) determinant conjugated to a variety of carriers. Skin reactions were measured at 4 and 24 hours. The results are shown in Table 4 and demonstrate that both antibody and cell mediated responses were elicited to the \( N_7 \) determinant. This observation is in agreement with previous work (Levy et al., 1972) which showed that similar conjugates elicited both types of skin reactions in guinea pigs immunized with O-Fd.

The specificity of the cell mediated response in guinea pigs immunized with \( N_7 \)-BSA was assessed using the leucocyte migration inhibition test. The results are presented in Table 5. In most respects, the results are analogous to those found with cells sensitized to O-Fd, in that \( N_4 \) through \( N_7 \) and O-Fd caused marked inhibition whereas N-M-Asp was not active and the aspartic acid modified peptide (GEE-\( N_7 \)) was weakly recognized.

A summary of the results obtained with antibody or immune cells is shown in Table 6.
DISCUSSION

The data summary in Table 6 clearly demonstrates that both antibodies and T cells from O-Fd immune animals recognize determinants within the N₆ peptide. However, the specificities are not identical. The lysine residue appears to be critical for antibody binding as modifications of the lysine (DNF-N₄) or elimination of it (N₄⁺) cause a loss of recognition. Conversely, DNP⁺N₇ and N₄ give positive migration inhibition. The addition of a hydrophobic group to the serine residue of N₄⁺ (N₄⁺-Bzl-Ser) restores antibody recognition and may indicate that the amino acids critical for recognition by antibodies are contained in N₄⁺, but lack sufficient bulk or hydrophobicity for binding. In contrast, the loss of migration inhibition to N₄⁺-Bzl-Ser demonstrates the important role of the serine in T cell recognition since the N₄ peptide inhibits migration well. Aspartic acid modified peptides (N-M-Asp and GEE-N₇) are considerably less able, if at all, to inhibit migration than their unmodified counterpart (N₇) implying the aspartic residue is necessary for binding to T cells. The N-M-Asp peptide is also less able to inhibit the complement fixation reaction which suggests that the aspartic residue plays a role in the determinant recognized by antibody, though not as important as in the determinant recognized by T cells. A pentapeptide (ala-tyr-lys-ile-ala) fragment from the amino determinant was unable to inhibit migration in previous work (Waterfield et al., 1974) emphasizing the importance of the aspartic and serine residues.

Of the peptides used in the migration inhibition tests with N₇-BSA immune guinea pig spleen cells, only N-M-Asp is not recognized. The migration inhibition found with GEE-N₇ is probably due to the creation of a new determinant on the immunogen N₇-BSA by coupling through the β-carboxyl of
the aspartic residue during conjugation to BSA. Recognition of $N_4$ suggests that the synthetic $N_7$ on BSA functions much the same as the amino determinant on 0-Fd.

The minimum size of an antibody binding determinant appears to be 4-6 residues in agreement with several reports (Young, Benjamine, Stewart and Leung, 1967; Benjamine, Young and Leung, 1968 a and b; reviewed by Goodman, 1969). In the present work the tetrapeptide, $N_4$, is consistently able to inhibit migration. Spitler et al (1969) found migration inhibition with a pentapeptide from tobacco virus protein.

The differences observed between antibody and T cell specificity may be due to two separate but overlapping determinants within $N_6$ or to the different natures of antibody and T cell receptors. If antibody and T cell receptors have different binding characteristics, a single modification may not have the same effect on the ability of the peptide to bind to these two receptors even if they both recognize identical determinants on the unmodified molecule. It is generally accepted that the antigen receptor on thymus independent lymphocytes (B cells) is antibody (reviewed by Vitetta and Uhr, 1975) and the specificity of B cells is probably very similar or identical to the antibody they produce (Makela, 1970). The nature of the receptor on T cells is still uncertain (Crone, Kock and Simonsen, 1972; Greaves, 1975; Munro and Taussig, 1975). Evidence from experiments where the antigen stimulated production of MIF by guinea pig cells was blocked by the addition of anti-$Y_1, F_2$ or $K$ implies that the receptor is immunoglobulin-like or closely associated with it on the cell surface (Goscicka, 1974). Also,
idiotypic determinants have been found on T cells (Binz, Wigzell, Ramseier and Lindenmann (1975)).

The ability of peptides to stimulate MIF production makes them very useful for studies of T cell specificity. Peptides can be synthesized and used directly without coupling them to other proteins or compensating for the presence and effect of other determinants. The relatively large quantities of peptides used to inhibit migration (0.05 μmole of peptide is equivalent to 280 μg O-Fd on the basis of molarity) probably reflects the fact that the peptides have little tertiary structure to maintain them in the configuration found in O-Fd. It has been shown that only a small percentage of peptides in solution have the same configuration they would have in the parent molecule (Crumpton and Small, 1967).

As found in other studies with small peptides, the peptides described here are unable to stimulate DNA synthesis in vitro in lymph node cells from either O-Fd or N₇-BSA immunized animals. One reported exception is a dodecapeptide from glucagon (Senyk et al, 1971) which is able to transform guinea pig cells. The ability of small peptides to generate MIF without stimulating DNA synthesis is consistent with reports that MIF production does not require DNA synthesis (Bloom, Gaffney and Jimenez, 1972; Rocklin, 1973). This dissociation between MIF production and transformation is unexplained, but suggests that two levels of activation can occur in T cells—activation with and without DNA synthesis. The report that cytotoxic cells can also be generated without DNA synthesis and cell division supports this hypothesis (MacDonald, Sordat, Cerottini and Brunner, 1975).

The peptides used in this study are not immunogenic unless coupled to a molecule such as BSA. It is apparent that N₇ and DNP (also a hapten)
do not act alike when conjugated to proteins. Both humoral and cellular immunity are directed to N7 while DNP is only recognized by antibodies. In animals immunized to N7-BSA, both N7 (when coupled to a heterologous molecule) and BSA individually elicit immediate and delayed skin reactions. The classical hapten-carrier phenomena is not expressed by N7-BSA. N7 can be recognized when coupled to diverse carriers such as BSA, PDG and PLL suggesting that shared determinants do not contribute significantly to either the antibody or T cell mediated response.

The inability of O-Fd to stimulate production of a useful titer of antibody in guinea pigs is unfortunate since the complement fixation tests had to be performed with rabbit serum. The effect of the species difference on the specificities is unknown.
TABLE 1

NAMES AND STRUCTURES OF SYNTHETIC PEPTIDES

<table>
<thead>
<tr>
<th></th>
<th>Structure</th>
<th>Composition</th>
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<td>ala - tyr - lys - ile - ala - asp - ser - CONHCH₂COOCH₂CH₃</td>
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</tbody>
</table>

An N₇ peptide diamidated with glycine ethyl ester.

N₄-Bzl-Ser

An N₄ peptide with intact O-benzyl protecting group.

NC₇

ser - leu - ala - tyr - asp - lys - ala

Leucine is substituted for isoleucine for identification purposes.
<table>
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<th>Amino Acid</th>
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<th>( N_3 )</th>
<th>( N_4 )</th>
<th>( N_5 )</th>
<th>( N_6 )</th>
<th>( N_7 )</th>
<th>( N-M )</th>
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</table>

- **x** - theoretical
- **+** - found
- **o** - molar ratios
- **xx** - disappearance of amino acid indicates modification
- ***** - lost during hydrolysis
# TABLE 3

## INHIBITION OF MIGRATION OF SPLEEN CELLS FROM O-Fd IMMUNE AND NON-IMMUNE GUINEA PIGS

<table>
<thead>
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<th>Antigen</th>
<th>O-Fd Immune</th>
<th>Unimmunized</th>
<th>t-test&lt;sup&gt;★&lt;/sup&gt;</th>
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</thead>
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<td>N&lt;sub&gt;7&lt;/sub&gt;</td>
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<td>0.99 ± 0.04</td>
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<td>N&lt;sub&gt;5&lt;/sub&gt;</td>
<td>0.73 ± 0.05</td>
<td>0.92 ± 0.07</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>N&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.71 ± 0.05</td>
<td>0.94 ± 0.06</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>N&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.90 ± 0.05</td>
<td>0.96 ± 0.08</td>
<td>&lt;.40</td>
</tr>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.91 ± .07</td>
<td>0.97 ± 0.09</td>
<td>&lt;.40</td>
</tr>
<tr>
<td>NC&lt;sub&gt;7&lt;/sub&gt;</td>
<td>0.93 ± 0.06</td>
<td>1.09 ± 0.08</td>
<td>&lt;.10</td>
</tr>
<tr>
<td>N-M-Asp</td>
<td>0.88 ± 0.06</td>
<td>1.14 ± 0.07</td>
<td>&lt;.005</td>
</tr>
<tr>
<td>GEE-N&lt;sub&gt;7&lt;/sub&gt;</td>
<td>0.86 ± 0.09</td>
<td>1.06 ± 0.08</td>
<td>&lt;.10</td>
</tr>
<tr>
<td>N&lt;sub&gt;4&lt;/sub&gt;-Bz1-Ser</td>
<td>0.92 ± 0.10</td>
<td>0.90 ± 0.10</td>
<td>&gt;.40</td>
</tr>
<tr>
<td>N8N 0.025 µmoles/ml</td>
<td>0.67 ± 0.110</td>
<td>0.87 ± 0.14</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>O-Fd 16 µg/ml</td>
<td>0.78 ± 0.03</td>
<td>1.06 ± 0.06</td>
<td>&lt;.0005</td>
</tr>
<tr>
<td>N&lt;sub&gt;7&lt;/sub&gt;-BSA 10 µg/ml</td>
<td>0.78 ± 0.05</td>
<td>1.01 ± 0.11</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>N&lt;sub&gt;7&lt;/sub&gt;-PDG 10 µg/ml</td>
<td>0.79 ± 0.05</td>
<td>0.98 ± 0.09</td>
<td>&lt;.025</td>
</tr>
<tr>
<td>N&lt;sub&gt;7&lt;/sub&gt;-PLL 10 µg/ml</td>
<td>0.73 ± 0.02</td>
<td>0.92± ± 0.09</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>DNP-N&lt;sub&gt;7&lt;/sub&gt;**</td>
<td>0.69 ± .13</td>
<td>0.92 ± .31</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

<sup>★</sup> - all peptides used at 0.05 µmoles/ml

<sup>★</sup> - mean ± standard error of the mean

<sup>★</sup> - probability calculated from the student's t-test that the ratio of migration in immune animals is different from that in unimmunized animals

<sup>★</sup> - from previously published work using animals immunized to a synthetic analogue of O-Fd (Waterfield et al, 1974).
TABLE 4

SKIN REACTIONS OBSERVED ON N\textsubscript{7}-BSA IMMUNIZED AND UNIMMUNIZED GUINEA PIGS

<table>
<thead>
<tr>
<th>Challenging Antigen**</th>
<th>N\textsubscript{7}-BSA IMMUNIZED</th>
<th>UNIMMUNIZED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arthus</td>
<td>Delayed</td>
</tr>
<tr>
<td>N\textsubscript{7}-BSA</td>
<td>15\textsuperscript{Q}</td>
<td>12</td>
</tr>
<tr>
<td>N8N\textsuperscript{*}</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>N\textsubscript{7}-PDG</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>N\textsubscript{7}-PLL</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Saline</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

\textsuperscript{Q} - average of 4 immunized and 4 unimmunized animals

\textsuperscript{*} - all antigens used at 50 \mu g/test

\textsuperscript{*} - two N\textsubscript{7} peptides bridged by 8 glycine residues in a linear manner (Kelly, Levy and Hull, 1973)

\textsuperscript{Q} - diameter of reaction in mm's
TABLE 5

INHIBITION OF MIGRATION OF SPLEEN CELLS FROM
N\textsubscript{7}-BSA IMMUNE AND NON-IMMUNE GUINEA PIGS

<table>
<thead>
<tr>
<th>Antigen</th>
<th>N-BSA Immune</th>
<th>Unimmunized</th>
<th>t-test\textsuperscript{\dagger}</th>
</tr>
</thead>
<tbody>
<tr>
<td>N\textsubscript{7}**</td>
<td>0.59 ± .05*</td>
<td>0.99 ± .04</td>
<td>&lt; .0005</td>
</tr>
<tr>
<td>N\textsubscript{5}</td>
<td>0.66 ± .07</td>
<td>0.92 ± .07</td>
<td>&lt; .01</td>
</tr>
<tr>
<td>N\textsubscript{4}</td>
<td>0.64 ± .09</td>
<td>0.94 ± .06</td>
<td>&lt; .005</td>
</tr>
<tr>
<td>N-M-Asp</td>
<td>0.97 ± .09</td>
<td>1.14 ± .07</td>
<td>&lt; .10</td>
</tr>
<tr>
<td>GEE-N</td>
<td>0.80 ± .07</td>
<td>1.06 ± .08</td>
<td>&lt; .025</td>
</tr>
<tr>
<td>O-Fd\textsuperscript{\textbullet}</td>
<td>0.75 ± .05</td>
<td>1.06 ± .06</td>
<td>&lt; .01</td>
</tr>
</tbody>
</table>

** - Peptides used at 0.05 µmole/ml
\textsuperscript{\textbullet} - used at 16 µg/ml
* - Mean ± standard error of the mean
\textsuperscript{\dagger} - See Table 3 for explanation
<table>
<thead>
<tr>
<th>Antigen or Peptide</th>
<th>O-Fd Immune</th>
<th>Leucocyte Migration</th>
<th>Leucocyte Migration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complement Fixation</td>
<td>Leucocyte Migration</td>
<td>Leucocyte Migration</td>
</tr>
<tr>
<td>N_7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N_6</td>
<td>+</td>
<td>+</td>
<td>ND*</td>
</tr>
<tr>
<td>N_5</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N_4</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N_3</td>
<td>-</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>N_2</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>NC_7</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>N-M-Asp</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>N_4-Bzl-Ser</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>GEE-N_7</td>
<td>ND</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DNP-N_7</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>O-Fd</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Not determined
LEGENDS

Fig. 1 A and B. Inhibition of the complement fixation reaction between O-Fd and homologous antisera by peptide analogues of the amino terminal determinant of O-Fd. Bars indicate the standard error of the means.
Fig. 1A
Fig. 1B

- N4-Bzl-Ser
- N-M-Asp
- DNP-N7
- NC7

% inhibition

μmoles peptide/test

0.003 0.006 0.012 0.025 0.050
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THE IMMUNE RESPONSE TO FERREDOXIN

II. CROSS REACTIVITY OF CELLS AND ANTISERA TO MODIFIED FERREDOXINS AND THE NATURE OF THE CELLS RESPONDING IN VITRO

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SUMMARY

The cross reactivity of sera from rabbits sensitized to performic acid oxidized ferredoxin (O-Fd) and of spleen cells from mice sensitized to O-Fd was analyzed using several chemically modified forms of ferredoxin in the complement fixation test and the in vitro lymphocyte stimulation assay. Only O-Fd and native ferredoxin (native-Fd) gave positive responses in both assays. Dinitrophenylated-O-Fd (DNP-O-Fd) and acid precipitated ferredoxin (TCA-Fd) were able to fix complement (C') but did not stimulate DNA synthesis in vitro. Ferredoxin alkylated with N-ethylmaleimide (NEM-Fd) induced cell transformation but fixed C' poorly. Carboxymethylated ferredoxin (CM-Fd) was unable to stimulate DNA synthesis and was marginally able to fix C'. Methylated-O-Fd (meth-O-Fd) was not recognized in either assay. The various ferredoxin preparations were tested for their ability to sensitize mice for use in the in vitro lymphocyte stimulation assay. Only O-Fd, NEM-Fd and native-Fd were capable of sensitizing lymphocytes for a proliferative response in vitro to the test antigens. This correlates with the observation that only these antigens were able to induce DNA synthesis in O-Fd sensitized lymphocytes.

The nature of the cells responding in vitro was examined by treating the cells with rabbit anti-mouse immunoglobulin and C' or rabbit anti-mouse brain associated θ and C'. The 24 hour response was found to be sensitive to both sera while the 120 hour response was sensitive only to the anti-θ sera.
The ability of the immune system to direct itself specifically against an antigen is well established. A number of investigations have been made to determine the ability of the immune response to discriminate between different antigens. Since the classic work of Landsteiner (1946) these studies have often been done with proteins in their native and chemically modified forms. A lack of humoral cross-reactivity between native and denatured forms of the same protein antigen has frequently been found. Antisera to native ribonuclease do not recognize performic acid oxidized ribonuclease (Brown, 1962; Brown, Delany, Levine and Van Vunakis, 1959).

Several other reports have demonstrated that while little or no humoral cross-reactivity was found, a high degree of recognition was seen at the level of delayed hypersensitivity. Antibodies to native lysozyme show very little reaction to S-carboxymethylated lysozyme (Gerwing and Thompson, 1968), but virtually complete reactivity was found in assays of cell-mediated immunity (Thompson, Harris, Benjamini, Mitchell and Nőbel, 1972). The affinity of anti-flagellin antibodies for flagellin was lost as the degree of acetoacetylation of the flagellin increased, while even highly substituted flagellin elicited delayed hypersensitivity in animals sensitized to native flagellin (Parish, 1971, a and b).

Using various chemically modified forms of bovine serum albumin, Schirrmacher and Wigzell (1972 and 1974) have demonstrated that thymus dependent lymphocytes (T cells) recognize a wider range of modified albumins than thymus independent lymphocytes (B cells) indicating different structural
requirements for recognition by these two cell types. Similar results were found by Parish (1972) with modified and heterologous erythrocytes. Two very similar proteins, egg white lysozyme and bovine α-lactalbumin, were highly cross reactive in cell mediated immune responses while demonstrating a complete lack of humoral reactions (Maron, Webb, Teitelbaum and Arnon, 1972).

The above findings have been taken to suggest that T cells recognize amino acid sequence (unaltered by modifications) while B cells recognize configuration (changed by modifications). We have examined this phenomena using the ferredoxin molecule which has been well characterized. Previous work has shown substantial antibody cross reactions between native, acid precipitated, S-carboxymethylated and performic acid oxidized ferredoxins (Nitz, Mitchell, Gerwing and Christensen, 1969).

The in vitro thymidine uptake assay has been thought to be a correlate of delayed hypersensitivity (Mills, 1966; Oppenheim, Wolstencroft and Gell, 1967; Meuvissen, van Alten and Good, 1969), presumably due to T cell transformation. However, Mugraby, Gery and Sulitzeanu (1974) have evidence that B cells are involved in the response. Since this in vitro method has been used in this study as an assay of cell-mediated immunity, the effects of T and B cell depletion on the thymidine uptake response to antigens and mitogens was examined. The cellular and humoral cross reactivities of several forms of ferredoxin were tested and the results are discussed in relation to the findings in the accompanying report (Gregerson et al, 1976).
MATERIALS AND METHODS

ANIMALS

Young adult albino rabbits were used to produce all the antisera. Mice of the strains DBA/2J and B6D2/J F1 were used for the in vitro stimulations.

ANTIGENS

Native ferredoxin (native-Fd) and performic acid oxidized ferredoxin (O-Fd) were prepared as described previously (Gregerson, et al, 1976). Acid precipitated ferredoxin (TCA-Fd) was made according to Tanaka, Nakashima, Mower and Yasunobu (1964). Dinitrophenylated O-Fd (DNP-O-Fd) was prepared by reacting O-Fd with 5% dinitrofluorobenzene in 10% aqueous sodium bicarbonate plus 20% ethanol for 2 days in the dark. The mixture was extracted several times with ether to remove unreacted dinitrofluorobenzene and then dialyzed. Methylated O-Fd (meth-O-Fd) was made as described by Fraenkel-Conrat and Olcott (1945) by suspending O-Fd in anhydrous methanol made to 0.1 M with concentrated hydrochloric acid. Esterification was allowed to proceed for 3 days in the dark followed by dialysis. The pH of meth-O-Fd solutions was kept below 6 until use to avoid alkaline hydrolysis of the esters (Ram and Maurer, 1959). Native-Fd was reduced with mercaptoethanol at pH 8 (1 µl/mg protein) and reacted with N-ethylmaleimide (2 equivalents/equivalent of sulfhydryl) at pH 6 for 4 hours followed by dialysis. The resulting alkylated product was called NEM-Fd. Native-Fd was S-carboxymethylated with iodoacetate as described by Battell, Zarkadas, Smillie and Madsen (1968) and dialyzed to give carboxymethylated Fd (CM-Fd). The various ferredoxins were analyzed and quantitated on a Beckman 120
amino acid analyzer after 18 hour hydrolysis in 6N HCl in evacuated vials.
The results are presented in Table 1.

IMMUNIZATIONS

Rabbits were immunized with O-Fd as described before (Gregerson et al., 1976). Mice were immunized twice at a 2-week interval with 35 µg of one of the various ferredoxins in 50% Freund's complete adjuvant.

COMPLEMENT FIXATION TESTS

These were done as described by Gerwing and Thompson (1968).

LYMPHOCYTE STIMULATIONS

In vitro microcultures were performed as described by Gregerson, Kelly and Levy (1975) except that mouse spleen cells were used in the present study. The non-immune controls for the DBA mice represent the pooled and averaged data from 12 separate experiments. Each experiment consisted of a minimum of nine determinations and usually more than 1 mouse.

CELL KILLING

Rabbit anti-mouse immunoglobulin (RaMIg) was prepared by immunizing a rabbit three times with 1 mg amounts of mouse immunoglobulin G (Miles Labs.) in complete Freund's adjuvant at 2 week intervals. The serum was collected several times, pooled and heat inactivated. Rabbit anti-brain
associated Θ (RaBAΘ) was prepared as described by Kelly, Kaye, Yoshizawa, Levy and Kilburn (1974). Guinea pig serum was absorbed with syngeneic mouse spleen cells and used as a source of complement (C'). All sera and C' were used at a 1:4 dilution in phosphate buffered saline (PBS). Cells were suspended in the diluted sera and C' at a concentration of $10^7$ cells/0.1 ml and incubated for 1 hour at 37°. After 3 washes in PBS the cells were used. Cells incubated with heat inactivated, spleen cell absorbed normal rabbit serum (NRS) and C' served as controls.
RESULTS

PARAMETERS OF LYMPHOCYTE STIMULATION BY ANTIGEN IN VITRO

Several experiments were done to determine the optimal conditions for the in vitro stimulation of DNA synthesis in spleen cells from O-Fd sensitized mice. The optimal dose of O-Fd was found to be 16 μg/ml in agreement with earlier work done using guinea pig cells (Gregerson et al., 1975). The results of the dose response experiments are presented in Table 2. The kinetics of the response using O-Fd at 16 μg/ml are shown in Table 3. A bi-modal response was found as seen previously using guinea pig cells under similar conditions. A few experiments done at 144 and 168 hours indicated the response was declining at these times and experiments were not routinely extended beyond 120 hours. In all cases the results are compared to those obtained from experiments with unsensitized cells by calculating the t-probabilities from the student's t-test. The relatively low stimulation indices observed in this study are probably due to the high background levels found in the medium controls.

THE NATURE OF THE CELLS RESPONDING IN VITRO

If the in vitro thymidine uptake assay as used in this study is a correlate of cell mediated immunity and dependent on T cells, pretreatment of the cells with RaBAo and C' should depress the response of O-Fd sensitized cells to O-Fd. The effect of treatment with RaMIg and C' (to kill immunoglobulin positive B cells) was also tested. Controls were done using C' and normal rabbit serum (NRS). The results in Table 4 summarize the effects of the
treatments on cell numbers. Since the C' and NRS were absorbed with syngeneic spleen cells to eliminate non-specific cytotoxicity, the cells lost during treatment with C' and NRS represent the loss due to the washing procedure and the death of various short lived splenic leucocytes during the incubation, washing and counting periods.

It has been shown that the responses of mouse spleen cells to concanavalin A (ConA) and lipopolysaccharide (LPS) are dependent on the presence of T and B cells, respectively (Andersson, Möller and Sjöberg, 1972). These mitogen responses were tested with each experiment and taken as an indication of the effectiveness of the treatments with RaBAθ, RaMIg and C'. It can be seen in Table 5 that the responses to ConA and LPS were greatly reduced by the respective antisera. The effects of these antisera on the response to O-Fd are presented in Table 6. Only those experiments in which the mitogen responses were substantially reduced by the antisera are included. The t-probabilities compare the means of treated and untreated (NRS and C') cells. The 24 hour response was found to be sensitive to both the RaMIg and RaBAθ indicating that B cells contribute to the response, but are dependent on the presence of T cells to do so. The 120 hour response was not significantly affected by the RaMIg treatment, but it was largely reduced by the RaBAθ sera. This result suggests that the 120 hour in vitro response is T cell mediated and not dependent on B cells. The response of the control cells treated with C' and NRS is somewhat depressed compared to the normal cells seen previously in Table 2. The cause for the decrease in this response is unknown.
The ability of O-Fd sensitized spleen cells to respond to several chemically modified forms of ferredoxin was examined using the in vitro lymphocyte stimulation assay. The O-Fd immunized mice were prepared as before. The data from 5 experiments summarized in Table 7 demonstrates that only NEM-Fd and native-Fd stimulated significant responses in cells from O-Fd sensitized mice. These antigens did not exhibit the bimodal response seen previously with O-Fd in Table 3, although the response to NEM-Fd was present at 24 hours.

The immunogenicity of the various ferredoxins was examined by using them to sensitize mice in the same manner as O-Fd was used. Spleen cells from these mice were tested in 4 experiments against several of the ferredoxins in vitro. Only NEM-Fd and native-Fd sensitized cells gave a proliferative response in vitro. Native-Fd and NEM-Fd stimulated DNA synthesis in NEM-Fd sensitized cells while native-Fd immunized cells only responded to native-Fd as seen in Table 8. Meth-Fd, TCA-Fd, CM-Fd and DNP-O-Fd did not elicit responses from any of the modified ferredoxin immune cells and did not sensitize cells for a response to any of the antigens. These results suggest that there may be a correlation between a molecule being able to stimulate an in vitro thymidine uptake response and to function as an immunogen. This phenomena has been reported previously (Levy, Hull, Kelly, Kilburn and Teather, 1972).

THE CROSS REACTIVITY OF ANTI-O-Fd ANTISERA

Using the complement fixation test, the cross reactivity of rabbit
anti-O-Fd antibodies was examined in several tests with modified ferredoxins.

Previous work using the complement fixation test demonstrated positive fixation with O-Fd, native-Fd, TCA-Fd and iodoacetamide alkylated ferredoxin (Nitz, et al., 1969). The results shown in Figure 1 indicate that DNP-O-Fd also fixes complement well while CM-Fd and NEM-Fd were only marginally active in this assay. The activity of meth-O-Fd was quite low.
DISCUSSION

The *in vitro* lymphocyte stimulation of mouse spleen cells by the antigen O-Fd, and the mitogens Con A and LPS, in terms of culture conditions (cell concentration and medium), kinetics and dose responses, was found to be remarkably similar to the responses of guinea pig cells as reported previously (Gregerson *et al.*, 1975).

Of particular interest was the bimodal nature of the *in vitro* response to O-Fd where two peaks of DNA synthesis were observed when the cells were labeled with tritiated thymidine at 24 and 120 hours of culture while virtually no response was found at 72 hours. Since the peak response to LPS was found when labeling was done at 24 hours, and LPS is considered to be a B cell mitogen for mouse cells (Anderson *et al.*, 1972), it seemed possible that B cells were contributing to the 24 hour response, either by DNA synthesis and/or by the production of a factor that could induce DNA synthesis in T cells. This problem was examined by pretreating the cells with RaMIg or RaBAO and C' to kill cells with immunoglobulin (B cells) (Raff, 1970) or Θ (T cells) (Golub, 1971) on their surfaces, respectively, and observing the effects these treatments had on the O-Fd, LPS and Con A responses. It was found that cells incubated with RaMIg and C' lost the 24 hour stimulation by O-Fd and LPS, but remained normally responsive to Con A. If the cells were preincubated with RaBAO and C' the responses to O-Fd and Con A were lost while the induction of DNA synthesis by LPS was slightly stronger than in untreated cells. These results suggest that:

i. the antisera were working properly as seen by the anti-Ig sensitivity of the LPS stimulation and the anti-BAO sensitivity of the Con A response.

ii. both T and B cells are required in significant numbers (it is assumed
that some cells escape killing by the antisera but obviously not enough to maintain reactivity) iii. either or both cell types may contribute to the DNA synthesized and detected by the assay, but both require the presence of the other cell type for induction of DNA synthesis, which may indicate the need for a cooperative response. The reduction of the 24 hour proliferative response by NRS and C', as compared to untreated cells, may be due to the loss of an adherent accessory cell, possibly macrophages, since the extra incubation and washings do reduce the numbers of adherent cells. Macrophages are required for stimulation in some \textit{in vitro} systems (Seeger and Oppenheim, 1970; Waldron, Horn and Rosenthal, 1973). This problem could be resolved by an investigation of the role of macrophages in the assay as performed in this study.

The response to O-Fd at 120 hours was not affected by preincubation with RaMIg or NRS and C', but was virtually eliminated by the RaBAO and C' treatment suggesting that the 120 hour response is, as classically taken, dependent on the presence of T cells. It is possible that the T cell response can induce DNA synthesis in B cells by the production of mitogenic factors, but the stimulation at 120 hours depends on the presence of adequate numbers of T cells. The 72 hour response was too low to show any affect by the sera. Mugraby, \textit{et al.}, (1974), using erythrocytes as the antigen have shown that both T and B cells are required for an \textit{in vitro} thymidine uptake response. These results are consistent with the assumption that the lymphocyte stimulation assay is a correlate of cell mediated immunity, but the contribution of B cells to the response is a factor that should be considered.

The experiments done to test the cross reactivity of the cell-mediated
response *in vitro* revealed that only three of the ferredoxins were active, either as immunogens or test antigens in culture. Only O-Fd was examined for its ability to stimulate antibody production. It was found that of the various ferredoxins, only O-Fd, NEM-Fd and native-Fd induced DNA synthesis *in vitro* in O-Fd sensitized cells. When tested for immunogenicity, NEM-Fd immune cells were stimulated by NEM-Fd and native-Fd—and less strongly by O-Fd. Native-Fd immune cells responded well to native-Fd, but not to the other ferredoxins. Previous work with a synthetic analogue of O-Fd, N-10-C (which contains only the two terminal determinants of O-Fd bridged by 10 glycine residues), has shown that a high degree of cross reactivity between N-10-C and O-Fd can be demonstrated in the lymphocyte stimulation assay, in immediate and delayed skin reactions, in the migration inhibition test and antibody production with either N-10-C or O-Fd functioning as the immunogen (Levy, Hull, Kelly, Kilburn and Teather, 1972; Kelly, Levy and Hull, 1973). In the present study, ferredoxins with modifications within the determinants of O-Fd (meth-O-Fd and DNP-O-Fd) did not stimulate DNA synthesis. Taken together, these results indicate that modifications which leave the two determinants of O-Fd unobstructed may not seriously affect their recognition by O-Fd sensitized cells. The lack of reactivity of those ferredoxins whose modifications were not in either of the determinants (TCA-Fd and CM-Fd) was probably due to structural changes which made the determinants inaccessible to the cells. The inability of some of the ferredoxins to immunize even when tested with the homologous antigen in culture is unexplainable at this time. It is interesting that NEM-Fd, which contains two hydrophobic N-ethylmalyl groups, functioned quite well as an immunogen and elicited a
stronger in vitro cellular response than the more highly charged O-Fd, but did not react well with anti-O-Fd sera. Somewhat similar results were observed by Parish (1971a and b) using acetoacetylated flagellin. He found that the acetoacetylation (which increases hydrophobicity and reduces charge) of flagellin produced a molecule which preferentially stimulated and elicited a cellular immune response but did not cross react with antibodies to the native molecule.

The cross reactivity of the rabbit anti-O-Fd antibodies was greater than was observed with the cell mediated response in vitro. Only meth-O-Fd was virtually unable to fix complement. Since this modification affects carboxyl groups, which are present in both the determinants of O-Fd and known to be important in the recognition of O-Fd, (Gregerson et al, 1976 and unpublished observations), this may account for its lack of reactivity. Strong cross reactivity was found with TCA-Fd and DNP-O-Fd, unlike the lymphocyte stimulation assay in which they were not recognized. NEM-Fd, recognized well in culture, fixed C' poorly. A number of reports have suggested that recognition by T cells is less specific than recognition by antibodies. This has not been seen in this study where the antibodies seemed to be less demanding. Also, in the previous section (Gregerson, et al, 1976), the migration inhibition assay demonstrated a high level of specificity. The nature and accessibility of the receptors, whether cell surface bound or free in solution, may have an effect on specificity. The possibility that the results presented here and in other studies may simply reflect the sensitivities of the assays must be considered. Because of the difficulty of obtaining anti-O-Fd sera in mice, rabbit anti-sera was used. The effect of this species difference is uncertain. Future work on specificity will involve an investigation of the specificity of B cell activation and T-T cell cooperation.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Theoretical</th>
<th>Native-Fd</th>
<th>O-Fd</th>
<th>Meth-O-Fd</th>
<th>NEM-Fd</th>
<th>TCA-Fd</th>
<th>DNP-O-Fd</th>
<th>CM-Fd</th>
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</thead>
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<td>8.8</td>
<td>8.9</td>
<td>8.0</td>
<td>10.0</td>
<td>9.7</td>
<td>7.6</td>
</tr>
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<td>1.1</td>
<td>0.9</td>
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<td>4.9</td>
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<td>1.6</td>
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<td>1.0</td>
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<td>7.8</td>
<td>3.8</td>
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<td></td>
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<td>carboxymethyl Cys</td>
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<td></td>
<td>3.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-succinyl Cys</td>
<td></td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$x^x$: molar ratios
$0^+$: produced by the oxidation of cysteine and cystine
$+^+$: derivative of cysteine produced by alkylation with iodoacetate
$q^q$: derivative of cysteine by alkylation with N-ethylmaleimide and subsequent acid hydrolysis
$^\star$: low values indicate modification
## TABLE 2
DOSE RESPONSE OF O-Fd SENSITIZED AND NON-IMMUNE DBA Spleen Cells to O-Fd IN VITRO**

<table>
<thead>
<tr>
<th>Immune Status</th>
<th>O-Fd ( \mu g/ml )</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>hours in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune</td>
<td></td>
<td>1.13 ± .03*</td>
<td>1.20 ± .09</td>
<td>1.36 ± .12</td>
<td>1.08 ± .12</td>
<td>72</td>
</tr>
<tr>
<td>Non-Immune</td>
<td></td>
<td>0.96 ± .09</td>
<td>0.96 ± .08</td>
<td>0.77 ± .19</td>
<td></td>
<td>72</td>
</tr>
</tbody>
</table>

\* probability calculated from the student's t-test that the response of the immune cells is different than that of the non immune cells

** represents 6 experiments, each done with a minimum of 2 mice and with 9 determinations per experiment.
TABLE 3

KINETICS OF THE IN VITRO RESPONSE OF DBA SPLEEN CELLS TO O-Fd AT 16 µg/ML**

<table>
<thead>
<tr>
<th>Immune Status</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune</td>
<td>1.60 ± .14</td>
<td>1.12 ± .07</td>
<td>1.35 ± .03</td>
<td>1.81 ± .18</td>
</tr>
<tr>
<td>Non-immune</td>
<td>1.01 ± .04</td>
<td>&lt; .005*</td>
<td>0.96 ± .08</td>
<td>1.10 ± .12</td>
</tr>
</tbody>
</table>

* t-probability - see Table 2 for explanation

** represents the data from 4 experiments and 9 determinations per experiment

hours before addition of label

hours in culture


<table>
<thead>
<tr>
<th></th>
<th>C' + NRS*</th>
<th>RaMIG + C'</th>
<th>RaBAO + C'</th>
<th>RaMIG + RaBAO + C'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>29%</td>
<td>56%</td>
<td>55%</td>
<td>72%</td>
</tr>
</tbody>
</table>

*NRS - normal rabbit serum

□ incubated simultaneously

★ loss of viable cells detected by trypan blue exclusion
## Table 5

**Effect of Treatment with RaBA\(\theta\) and RaMI\(g\) Plus C' on the Responses of DBA Spleen Cells to ConA and LPS**

<table>
<thead>
<tr>
<th>Hours</th>
<th>NRS and C'</th>
<th>RaMI(g) and C'</th>
<th>RaBA(\theta) and C'</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>89,913 ± 9571*</td>
<td>89,225 ± 18,873</td>
<td>4525 ± 2479</td>
</tr>
<tr>
<td>448</td>
<td>114,568 ± 24,754</td>
<td>8388 ± 3723</td>
<td>14,633 ± 9247</td>
</tr>
<tr>
<td>72</td>
<td>23,388 ± 3295</td>
<td>5864 ± 2699</td>
<td>6890 ± 3549</td>
</tr>
<tr>
<td>96</td>
<td>3243 ± 5308</td>
<td>-387 ± 1534*</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>11276 ± 3283</td>
<td>5864 ± 2699</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>-387 ± 1534*</td>
<td>5864 ± 2699</td>
<td></td>
</tr>
</tbody>
</table>

**ConA 2 \(\mu g/ml\)**

<table>
<thead>
<tr>
<th>Hours</th>
<th>NRS and C'</th>
<th>RaMI(g) and C'</th>
<th>RaBA(\theta) and C'</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>57,995 ± 8443*</td>
<td>8888 ± 1523</td>
<td>62,352 ± 8096</td>
</tr>
<tr>
<td>48</td>
<td>38,946 ± 10,665</td>
<td>-630 ± 1283</td>
<td>29,979 ± 2677</td>
</tr>
<tr>
<td>72</td>
<td>13,929 ± 3218</td>
<td>-630 ± 1283</td>
<td>29,979 ± 2677</td>
</tr>
<tr>
<td>96</td>
<td>-1667 ± 3374</td>
<td>474 ± 1004</td>
<td>7647 ± 2801</td>
</tr>
<tr>
<td>120</td>
<td>511 ± 2318</td>
<td>474 ± 1004</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>-1783 ± 1608</td>
<td>474 ± 1004</td>
<td></td>
</tr>
</tbody>
</table>

\*stimulated cpm minus unstimulated controls

\* responses below the unstimulated background are expressed as negatives

**LPS 16 \(\mu g/ml\)**

<table>
<thead>
<tr>
<th>Hours</th>
<th>NRS and C'</th>
<th>RaMI(g) and C'</th>
<th>RaBA(\theta) and C'</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>89,913 ± 9571*</td>
<td>89,225 ± 18,873</td>
<td>4525 ± 2479</td>
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<tr>
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</tr>
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<td>96</td>
<td>3243 ± 5308</td>
<td>-387 ± 1534*</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>11276 ± 3283</td>
<td>5864 ± 2699</td>
<td></td>
</tr>
<tr>
<td>144</td>
<td>-387 ± 1534*</td>
<td>5864 ± 2699</td>
<td></td>
</tr>
</tbody>
</table>

\*hours in culture before labeling

**represents the pooled results of 6 experiments, each done on a minimum of 4 mice with at least 9 determinations per experiment**
### TABLE 6

**EFFECT OF TREATMENT WITH RaBA\(\theta\) AND RaMIg PLUS C' ON THE RESPONSE OF O-FD IMMUNE DBA SPLEEN CELLS TO O-Fd**

<table>
<thead>
<tr>
<th>Hours</th>
<th>C' and NRS</th>
<th>RaMIg and C'</th>
<th>RaBA(\theta) and C'</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1.36 ± 0.08</td>
<td>1.08 ± 0.06</td>
<td>&lt;.01*</td>
</tr>
<tr>
<td>72</td>
<td>0.99 ± 0.09</td>
<td>0.95 ± 0.08</td>
<td>&gt;.25</td>
</tr>
<tr>
<td>120</td>
<td>1.70 ± 0.17</td>
<td>1.70 ± 0.29</td>
<td>&gt;.25</td>
</tr>
</tbody>
</table>

* t-probability that the response is different than the response of cells treated with NRS and C'

\(\uparrow\) hour in culture prior to labeling

** represents the pooled results of 6 experiments, each done on a minimum of 4 mice with at least 9 determinations per experiment

\(\uparrow\uparrow\) stimulation index ± SEM
### TABLE 7

RESPONSES OF O-Fd SENSITIZED AND UNSENSITIZED SPLEEN CELLS FROM DBA MICE TO MODIFIED FERREDOXIN ANTIGENS AT 16 µg/ml**

<table>
<thead>
<tr>
<th>Hours</th>
<th>NEM-Fd</th>
<th>Meth-O-Fd</th>
<th>CM-Fd</th>
<th>TCA-Fd</th>
<th>Native-Fd</th>
<th>DNP-O-Fd</th>
<th>Immune Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1.79 ± .32</td>
<td>1.00 ± .03</td>
<td>0.84 ± .08</td>
<td>1.18 ± .17</td>
<td>1.13 ± .13</td>
<td>1.05 ± .04</td>
<td>immune</td>
</tr>
<tr>
<td>72</td>
<td>2.19 ± .55</td>
<td>0.74 ± .09</td>
<td>0.95 ± .26</td>
<td>0.99 ± .14</td>
<td>1.32 ± .47</td>
<td>0.83 ± .26</td>
<td>immune</td>
</tr>
<tr>
<td>120</td>
<td>2.99 ± .68</td>
<td>0.97 ± .11</td>
<td>0.90 ± .18</td>
<td>1.16 ± .13</td>
<td>1.94 ± .31</td>
<td>1.04 ± .28</td>
<td>immune</td>
</tr>
<tr>
<td>120</td>
<td>0.86 ± .17</td>
<td>0.98 ± .166</td>
<td>1.03 ± .12</td>
<td>1.18 ± .18</td>
<td>1.04 ± .07</td>
<td>0.73 ± .09</td>
<td>non-immune</td>
</tr>
</tbody>
</table>

- NEM-Fd: 0.08 ± .05
- Meth-O-Fd: 0.00 ± .05
- CM-Fd: 0.08 ± .05
- TCA-Fd: 0.08 ± .05
- Native-Fd: 0.08 ± .05
- DNP-O-Fd: 0.08 ± .05

* t-probabilities

** represents the pooled results of 5 experiments and 9 determinations per experiment
TABLE 8
IN VITRO RESPONSES OF B6D2/J F1 MICE IMMUNIZED TO MODIFIED FERREDOXINS

<table>
<thead>
<tr>
<th>Hours⁹</th>
<th>NEM-Fd*</th>
<th>O-Fd</th>
<th>Native-Fd</th>
<th>Immune status</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1.26 ± .18&lt;²</td>
<td>1.25 ± .25</td>
<td>1.11 ± .11</td>
<td>NEM-Fd immune</td>
</tr>
<tr>
<td>24</td>
<td>1.16 ± .23</td>
<td>0.98 ± .04</td>
<td>1.07 ± .04</td>
<td>Non-immune</td>
</tr>
<tr>
<td>72</td>
<td>1.16 ± .18</td>
<td>1.08 ± .14</td>
<td>1.07 ± .18</td>
<td>NEM-Fd immune</td>
</tr>
<tr>
<td>72</td>
<td>0.80 ± .22</td>
<td>0.97 ± .06</td>
<td>0.70 ± .07</td>
<td>Non-immune</td>
</tr>
<tr>
<td>120</td>
<td>2.13 ± .54</td>
<td>1.50 ± .45</td>
<td>2.44 ± .64</td>
<td>NEM-Fd immune</td>
</tr>
<tr>
<td>120</td>
<td>0.75 ± .15</td>
<td>1.08 ± .09</td>
<td>0.96 ± .10</td>
<td>Non-immune</td>
</tr>
<tr>
<td></td>
<td>&lt; .01</td>
<td>&lt; .10</td>
<td>&lt; .05</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>1.19 ± .14</td>
<td>1.04 ± .06</td>
<td>1.24 ± .14</td>
<td>Native-Fd immune</td>
</tr>
<tr>
<td>24</td>
<td>1.16 ± .23</td>
<td>0.98 ± .04</td>
<td>1.07 ± .04</td>
<td>Non-immune</td>
</tr>
<tr>
<td>72</td>
<td>0.67 ± .07</td>
<td>0.98 ± .16</td>
<td>1.04 ± .11</td>
<td>Native-Fd immune</td>
</tr>
<tr>
<td>72</td>
<td>0.80 ± .22</td>
<td>0.97 ± .06</td>
<td>0.70 ± .07</td>
<td>Non-immune</td>
</tr>
<tr>
<td>120</td>
<td>1.32 ± .27</td>
<td>1.14 ± .19</td>
<td>1.87 ± .55</td>
<td>Native-Fd immune</td>
</tr>
<tr>
<td>120</td>
<td>0.75 ± .15</td>
<td>1.08 ± .09</td>
<td>0.96 ± .10</td>
<td>Non-immune</td>
</tr>
<tr>
<td></td>
<td>&lt; .05</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** represents the results from 4 experiments and nine determinations per experiment

* t-probability

⁹ hours in culture prior to 18 hour labeling

x stimulation index ± SEM

* all ferredoxin antigens used in culture at 16 μg/ml
Fig. 1. Complement fixation reactions of pooled antisera against O-Fd at a 1:40 dilution with several preparations of ferredoxin: ■—■, TCA-Fd; ▲—▲, DNP-O-Fd; △—△, NEM-Fd; ●—●, O-Fd; □—□, CM-Fd; ○—○, meth-O-Fd.
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CONCLUSION

Examination of the results of the \textit{in vitro} lymphocyte stimulation assay revealed that the responses of guinea pig and mouse cells to the mitogens Con A and LPS and the antigen O-Fd are very similar in terms of dose response and kinetics. The LPS responses of both species were sensitive to treatment with anti-immunoglobulin and complement although the stimulation of mouse spleen cells by LPS was reduced to a much greater degree than was the response of guinea pig cells.

Of particular interest was the specific response \textit{in vitro} to the antigen, O-Fd. The ability of O-Fd to stimulate a proliferative response in serum free medium with and without mercaptoethanol was tested using guinea pig lymph node cells. Both of these media supported an early response which peaked at 48 hours and rapidly declined. The addition of foetal calf serum (FCS) to either of these media resulted in a bimodal response to O-Fd with peak stimulations occurring at 24 and 96 to 120 hours. O-Fd sensitized mouse spleen cells also gave a bimodal response to O-Fd in medium with FCS. Peak responses were found at 24 and 120 hours as seen with the guinea pig cells. By pretreating the mouse spleen cells with anti-immunoglobulin and complement or anti-brain associated \( \Theta \) and complement, it was found that the 24 hour response required the presence of both T and B cells in large numbers while the 120 hour response required only T cells. These results corroborate the T cell dependency of the \textit{in vitro} lymphocyte stimulation assay and the validity of its use as a correlate of cell mediated immunity.

The specificity of the cellular and humoral immune responses to several forms of ferredoxin were tested using the lymphocyte stimulation
assay to measure cell mediated immunity and the complement fixation test to
detect antibody reactivity. Only one modified ferredoxin, the methyl
estérfié form (meth-O-Fd) was not recognized by the anti-O-Fd sera. This
lack of recognition was probably a result of meth-O-Fd being modified in
both of the determinants present on O-Fd. Other ferredoxins were at least
moderately recognized by the anti-O-Fd sera. Only ferredoxins not modified
in either determinant (O-Fd, native-Fd and NEM-Fd) were able to stimulate
DNA synthesis in vitro in O-Fd sensitized mouse spleen cells. These results
indicate that in the ferredoxin system, antibodies are more cross reactive
than is the cellular immune response. This is in contrast to most published
work in other antigen systems and may reflect the antigenic nature of O-Fd
which contains only two determinants, one on each terminus of the molecule.

It was of interest to note that only ferredoxins which stimulated DNA
synthesis in vitro were immunogenic.

The specificity of the anti-O-Fd response was also studied in greater
detail using the amino terminal determinant of O-Fd. These studies
indicated that both antibodies and T cells are highly specific, but differ
in their specificity requirements. There are several possible explanations
of the results found using the peptide analogues of the amino determinant:
either i.) the receptors of T cells and the antibodies have different
characteristics but react to the same determinant, or ii.) the T cell
receptors and antibodies are similar but are reacting to overlapping
determinants, or iii.) both possibilities occur.

The more definitive results obtainable using small monovalent peptide
determinants to assay immune specificity indicate that specificity studies
done by modification of an entire antigen may be misleading, as such
modifications greatly affect molecular conformation and may simply decrease the accessibility of some determinants rather than effecting a change to the determinants themselves. The results of studies in this thesis using modified ferredoxin antigens indicate that antibodies are more cross reactive than T cells. However, this is not consistent with the results of the peptide studies which show that both responses are specific. This supports the contention that the indiscriminate modification of proteins may not provide useful antigens for specificity studies. The peptide studies presented here suggest that T cells and antibodies have similar specificities in that neither seems more cross reactive than the other, however, their manner of recognition may be different.