STUDIES ON ANTIGEN BINDING CELLS INVOLVED IN

CELLULAR IMMUNITY TO FERREDOXIN PEPTIDES

by

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ABSTRACT

Previous studies with conjugates containing the NH_2-terminal and COOH-terminal antigenic determinants of oxidized ferredoxin from C. pasteurianum indicated a need for at least two determinants to stimulate DNA synthesis in sensitized lymphocytes. This suggested a mechanism involving cell cooperation, a possibility which has been investigated here by selectively inactivating cells binding one or the other of the determinants.

Cells from immunized guinea pigs were tested in vitro for their capacity to bind antigen or to be stimulated by it before and after "antigen suicide" with radioiodinated conjugates containing the NH_2-terminal or COOH-terminal determinants of oxidized ferredoxin. A microculture system for assessing antigen induced stimulation of ^{3}H-thymidine uptake by lymphocytes was developed for this work.

The data show that:

1) Lymphocytes from unimmunized guinea pigs bind both NH_2-terminal and COOH-terminal determinants at a frequency of about 10^{-4}. In immune animals the proportion of antigen binding cells increased about 4-6 fold. The frequency of cells binding the determinants depends markedly on the specific activity of antigens employed.

2) Both T and B lymphocytes bind the antigenic determinants from oxidized ferredoxin.

3) Specific inactivation of cells binding either determinant was

achieved by antigen suicide with ^{125}I-NH_2-terminal or ^{125}I COOH-terminal s-BSA conjugates.

4) Synergy occurs between the NH_2-terminal binding cells and COOH-terminal binding cells in the proliferative response of sensitized lymph node cells challenged with oxidized ferredoxin in vitro. Evidence from B cell depletion studies indicates that this is a T cell-T cell interaction.

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ABBREVIATIONS

IgG   Immunoglobulin of heavy chain antigenic type γ (gamma)

IgM   Immunoglobulin of heavy chain antigenic type μ (mu)

DNP-POL   Dinitrophenylated polymerized flagellin

ABA-Tyr   Azobenzenearsonate-L-Tyrosine

DNA   Deoxyribonucleic acid

PDG   Poly-D-glutamic acid

MIF   Migration inhibition factor

Budr   Bromodeoxyuridine

GVH   Graft versus host

MLC   Mixed leukocyte culture

CML   Cell mediated lympholysis

$N_{10}C$ The NH_2-terminal haptenic peptide and the COOH-terminal hap-
           tenic peptide of C. pasteurianum ferredoxin bridged by 10
           glycine residues

OFD   Oxidized ferredoxin

$C_{mal}C$ The symmetrical synthetic molecule formed by bridging two
           COOH-terminal peptides with a glycine-malonic acid bridge

KLH   Keyhole limpet hemocyanin

R.P.M.I.   Medium number 1640 from the Roswell Park Memorial Institute
1640

PBS   Phosphate buffered saline*

TCA   Trichloracetic acid

s-BSA   Succinylated bovine serum albumin

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* Saline and phosphate buffered saline of isotonic strength.

PBS: NaCl   8 gm/l KH_2PO_4 0.2 gm/l

        KCl   0.2 gm/l $Na_2HPO_4 \cdot 7H_2O$ 2.17 gm/l

Abbreviations (Continued)

N-sBSA   The NH_2-terminal haptenic peptide covalently linked to suc-
          cinylated BSA in a ratio of approximately 20:1 (peptide:
          sBSA)

C-sBSA   The COOH-terminal haptenic peptide covalently linked to suc-
          cinylated BSA in a ratio of approximately 22:1 (peptide:
          sBSA)

EDCI   1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl

FCS   Fetal calf serum

PPD   Purified protein derivative (from Mycobacterium tuberculosis)

PHA   Phytohemagglutinin (obtained from Phaseolus vulgaris)

ConA   Concanavalin A

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INTRODUCTION

I   Clonal Selection Theory

According to Burnet's clonal selection theory (14), immune responses involve the triggering of precommitted clones of antigen sensitive cells and their subsequent development to give a specific humoral or cellular immunity. Central to this theory are the following points:

(1) Individual lymphocytes are precommitted to synthesis of antibody of a single specificity and are restricted to production of this single molecular species.

(2) There are sites present on the cell surface which recognize a given antigen and initiate production of a specific antibody.

(3) The antigen recognition site is linked to the antibody it controls, that is, individual antigen reactive cells constitutively produce antibody which reacts with antigen and triggers the cell to proliferate and produce large quantities of antibody.

(4) The antibody response is due in part to proliferation of the cells producing antibody. That is, the cell specialization is heritable (clonal).

Hypotheses regarding generation of diversity of precommitted lymphocytes be they somatic generation (12,13,14,15,19,36,61,116) or germ-line theories (32,33,54) are compatible with clonal selection. The generation of immune diversity will not be discussed here as the clonal selection theory and the ideas central to this thesis involve the immune

system after it has gained diversified competence.

II Antigen Binding Cells

Naor and Suliztneau (79) originally tested Burnet's hypothesis that lymphoid cells react specifically with antigen. Using autoradiographic techniques they found a small number of mouse spleen cells bound radio-iodinated bovine serum albumin (BSA) in vitro. Byrt and Ada observed that a similar number of cells bound radioiodinated flagellin and hemocyanin (17). Both groups tested antigen binding under conditions that inhibited phagocytosis. As predicted by the clonal selection theory, only a small proportion of lymphoid cells bound any given antigen. Ada (1) has reviewed the data on radiolabelled antigen binding in lymphoid populations. The major observations are:

(1) The binding reaction is usually complete within 60 minutes at 4 C although a time dependence is evident (1).

(2) A majority of cells labelled are small lymphocytes although medium lymphocytes, macrophages and plasma cells are occasionally labelled (79).

(3) Ultrastructural analysis showed that antigen (in this case ferritin) was present on the cell membrane and was bound in a "patchy" fashion on the lymphocyte surface (68).

(4) The number of lymphocytes binding any one antigen is a small proportion of the total cell number, usually in the order of 10^{-4} - 10^{-5} in unprimed populations (1).

(5) In immune animals, the proportion of antigen binding cells increased, usually about ten-fold (80,55,2).

Can one equate the binding cells in a virgin population to the precursors of clones as postulated by Burnet? On the basis of numbers alone, no conclusion can be reached. There appear to be too many binding cells for a given antigen. At a frequency of 10^{-4}, binding cells for only 10^4 different antigens are allowed. It is argued however that this figure (10^{-4}) includes cross-reacting cells. Also, a hierarchy of binding cells for a given antigen exists. That is, some cells bind antigen at low concentrations and bind progressively more antigen as the concentration increases. Other cells bind antigen only at relatively high concentrations (1). This observation can be explained by random generation of receptor diversity. As the concentration of antigen increases, there will be more cells that bind antigen due to less stringent specificity requirements. The main question regarding the significance of antigen binding cells concerns their identity with immuno-competent cells.

III Functional Significance of Antigen Binding Cells

An increasing body of evidence points to the conclusion that at least a portion of the cells binding a given antigen are immunocompetent:

(1) Wigzell and Andersson used antigen coated glass bead columns to selectively remove immunocompetent cells from a lymphoid population while reactivity against an unrelated antigen remained intact (137). Recovery of the depleted cells was unsuccessful.

(2) Edelman et al (37, 103, 104), Truffa-Bachi and Wofsy (125) and Wofsy et al (138) achieved specific cell elimination using immunoadsorbant techniques, and recovered the antigen binding cells.

(3) Deletion of specific immunocompetent cells was reported by Dutton and Mishell (35) using a "hot-pulse" technique. In vitro incubation of mouse spleen cells stimulated by sheep erythrocytes and given a pulse of high specific activity tritiated thymidine, removed anti sheep erythrocyte antibody forming cells while leaving the response to burro erythrocytes intact.

(4) Lymphocytes stimulated with the protein antigen streptokinase-streptodornase varidase (SK-SD) were inactivated using Budr (5-Bromo-2-deoxyuridine) and light (139). Zoschke and Bach found that thymidine uptake was deleted when SK-SD was the stimulating antigen, while the response to unrelated antigens was left intact.

(5) Diener (31), using cells separated by velocity sedimentation showed that a large proportion of antigen binding cells were induced by antigen to differentiate into larger cells. These

larger lymphocytes followed the distribution profile of immuno-
competence in a cell population. The significance of the
antigen binding cells not stimulated by antigen is uncertain.

The relationship between immune competence and antigen binding cells
is further complicated by a functional division in the immune system.
Introduction of antigen into an animal may stimulate humoral immunity,
cell-mediated immunity, or both, depending on the nature of the antigen
and method of administration. The division in the immune system is
attributable to two functionally distinct classes of lymphocytes:

B Lymphocytes (Bursa or bone marrow derived) are associated with
humoral immunity and are the progenitors of antibody forming cells
(18,24).

T Lymphocytes (Thymus processed or thymus derived) mediate cellular
immunity and also function as "helper" cells in the production of
antibody (18).

Katz and Benacerraff (57) have reviewed the specificity of immune responses
attributed to T and B lymphocytes.

IV <u>Antigen Receptors on Lymphocytes</u>

The recognition of antigen molecules by lymphoid cells is the first step required for initiating the complex events involved in the immune response. The chemical nature of cell surface antigen receptors has recently been under intense investigation in several laboratories. Originally, Sell and Gell (111) observed blast cell formation in cultures treated with antiimmunoglobulin antibody, suggesting that immunoglobulin is present on the cell surface and that interaction with this "receptor" in some way stimulates lymphocytes to proliferate. The presence of immunoglobulin on the surface of B lymphocytes has been demonstrated quite conclusively:

(1) The proportion of immunoglobulin positive lymphocytes in various tissues was examined using radioiodinated or fluorescent anti-immunoglobulin antibodies (89,92,128). Spleen, lymph nodes, peripheral blood, and thymus lymphocytes contained 45-60%, 10-30%, 10-25% and less than 1% labelled lymphocytes respectively, figures that correspond to the proportion of B cells in these tissues (93).

(2) Stronger evidence that immunoglobulin carrying cells are B cells was found by comparing cells from thymectomized, irradiated, bone marrow reconstituted mice and control untreated mice (130). No immunoglobulin negative lymphocytes were found in the "B reconstituted" animals.

(3) Using class specific anti-immunoglobulin antibody, the surface immunoglobulin was shown to be mainly IgM and IgG in the mouse (127) IgM in the rabbit and man, and IgG in the guinea pig (87).

The nature of the T cell receptor is still controversial:

(1) Raff, using fluorescein coupled anti IgG, could not demon-
strate immunoglobulin on the surface of murine lymphocytes
possessing the alloantigen Theta (Θ) (93).

(2) Perkins et al using ultastructural autoradiography (90) and
Karnovsky using freeze-etching techniques (56) failed to
detect surface immunoglobulin on T lymphocytes.

(3) Using lengthy autoradiographic exposure times, Bankhurst et al
labelled thymus and thymus derived lymphocytes with radioio-
dinated anti-light chain antibody (8).

(4) Marchalonis et al, isolated 7S IgM from the surface of human
and murine thymus lymphocytes having iodinated surface proteins
by the lactoperoxidase iodination technique (69). Uhr et al,
however, were unable to reproduce these results using similar
methodology (127).

The difficulty in detecting immunoglobulin on T cells has been ex-
plained in several ways:

(1) Extended exposure times needed for detection of immunoglobulin
on T cells in autoradiographic procedures suggests that T
lymphocytes possess a much lower density of immunoglobulin
than B cells (8).

(2) Lack of T cell labelling with anti-immunoglobulin antibody
reflects relative accessibility between T and B lymphocyte
surface immunoglobulin (69).

(3) The T cell receptor may not be a conventional immunoglobulin

Mitchison has proposed the term IgX (75) and Nossal IgT (83).

(4) Feldmann (40) has recently shown the existence of an Ig-antigen complex which is shed from T cells. Cone et al. (21) showed identity of the Ig in this complex with 7S IgM, using lactoperoxidase iodination techniques. Receptor shedding may cause difficulty in surface detection.

Although a paucity of evidence exists showing T cell surface immunoglobulin directly, the experiments of Marchalonis (69), Feldmann (40) and Cone et al. (21), indicate that immunoglobulin is present on T cell surfaces. Since the most convincing evidence was gained using the extremely sensitive surface iodination technique, perhaps other labelling techniques, which depend on using anti-immunoglobulin antibody, are inadequate.

Demonstration of surface Ig doesn't necessarily assign it a role in the immune response. However, a number of studies indicate that immunoglobulin is, in fact, the antigen receptor on both T and B lymphocytes:

(1) Radiolabelled antigen binding reactions are inhibited by anti-immunoglobulin antibodies (2, 134).

(2) The in vitro humoral response to several antigens is inhibited by anti-light chain sera (63).

(3) In vivo immune response to several antigens is inhibited in mice reconstituted with anti-μ treated spleen cells (134).

(4) Rosette formation in T cell enriched populations is inhibited by anti μ and anti light chain sera (63).

(5) Graft versus host reactions are inhibited by anti-light chain antisera (70).

(6) Specific removal of T lymphocytes using "antigen-suicide" techniques is inhibited by anti-immunoglobulin sera (9, 100). Functional inhibition studies implicating immunoglobulin as T cell antigen receptor are reviewed by Simonsen (23).

The precise events that lead to lymphocyte triggering after antigen binding to cell receptors are largely unknown. Lipid-protein surface membrane components undergo conformational alteration after antigen-receptor interaction and it is hypothesized that these allosteric changes are involved in lymphocyte activation (27). There appears to be a relationship between the localization of antigen and the activation of lymphocytes, by the mobilization of surface receptors to form patches or caps (56,95,30).

V   Cell Cooperation in Immune Responses

The elucidation of the mechanism of lymphocyte stimulation by antigens is complicated by the apparent interaction of T and B lymphocytes. Studies on this cooperative process have been concerned almost entirely with production of humoral antibody, with the following observations emerging:

(1) Both thymus cells and bone marrow-derived cells are required to restore immune capacity to an irradiated animal (18,24).

(2) In hapten-carrier experiments, both thymus-derived cells primed to an immunogenic carrier and bone marrow cells primed to hapten are required for secondary anti hapten response to the hapten-carrier conjugate (76,77).

(3) Antigen specificity exists in both thymus-derived and bone-marrow-derived populations (9,76,77).

(4) In some instances, intact living thymus cells and bone marrow cells are needed for antibody production (24), while in others some antigens are thymus independent and stimulate B cells directly (72).

(5) Both thymus-derived and bone marrow-derived cells have a radiosensitive component in their restorative capacity in cell cooperation (24).

(6) Both hapten and carrier moieties must be present on the same molecule in order to obtain an anti hapten response upon antigen challenge (76,77).

Several theoretical models have been proposed to account for these data:

Two Cell Hypotheses

(1) <u>The "Local Concentration" Hypothesis</u>. Mitchison (75) suggested that initially the T cells react with antigenic determinants present on the carrier molecule, localizing haptenic determinants for presentation to the hapten-reactive B cell via an "antigen bridge" between the cells. The T cell fulfills a passive role acting as a helper cell presenting the antigen to the B cell in the required concentration or spatial arrangement.

(2) <u>The "Cross-Linking" Hypothesis</u>. Bretcher and Cohn (11) postulated that union between B cells and antigen is necessary for activation but that cross-linking of antigen is required. An antibody to a different determinant on the antigen must react with the antigen adsorbed to the B cell. The antigen specific receptors, either on the T cells or after secretion may cross-link the antigen molecules on the B cells.

(3) <u>The "Factor" Hypothesis</u>. Dutton <u>et al</u> (34) suggested that both T and B cells bind antigen. After antigen binding, T cells become activated and secrete a non-specific stimulus to the B cells, which become competent to respond to antigen.

Schimpel and Wecker, using allogeneic T cell stimulation, found non-specific soluble T cell factors which enhanced the B cell response to any antigen capable of stimulating them (106). Feldmann and Nossal (41) substantiated this report using syngeneic mouse cells <u>in vitro</u>. Non-specific T cell

factor was produced providing a strong T cell response was generated.
Recent work by Feldmann and his colleagues favors a 3 cell collaborative
system:

(1) Using metabolic inhibitors, it was found that virgin T cells
must divide before becoming functional helper cells (40).

(2) Cell co-operation occurred with T cells separated from B cells
by a cell impermeable nucleopore membrane (39).

(3) Adherent cells were required for antibody response to hapten-
protein antigens that require T and B lymphocyte co-operation.
Response to a T cell-independent antigen (DNP-POL) was not
diminished by removal of adherent cells (41).

(4) A specific "collaborative factor" was produced by activated
T cells, and became macrophage bound. These macrophages
retained sufficient collaborative factor to trigger B cells
into antibody production (40).

(5) Treatment of collaborating macrophages with anti kappa chain
or anti μ chain antisera abrogated the antibody response (40).

The last observation led to the view that T cells had produced an IgM-
like receptor molecule, which when complexed to antigen became liberated
from the T cell surface and attached to macrophages. The monomeric
surface IgM found on human and mouse thymocytes by Marchalonis et al (69)
was compared to isolated collaborative factor (21). Complete identity
was found. The tendency of T and B cell surface immunoglobulin to bind
to macrophages was then investigated by Cone et al., using surface radio-

iodination techniques (21). T cell derived IgM product bound to mac-rophages while B cell surface immunoglobulin did not.

Three Cell Hypotheses

(1) T Cell-Macrophage-B Cell

Feldmann (40) formulated the following model: Activated thymus cells release IgM-like molecules complexed with the inducing antigen. This shed complex binds to the macrophage surface and a concentrated depot of antigenic determinants is formed. The macrophages present the deter-minants to B cells which are then triggered.

(2) Macrophage-T Cell-B Cell

An alternative three cell model was proposed by Katz and Unanue (58). Antigen is initially bound by macrophages which present the anti-gen to T cells. The T cells are then activated and in turn trigger B cells.

The three cell models are particularly attractive in that they suggest mechanisms governing control of the antibody response, that is, regulation by macrophage mediation. Regulation of humoral antibody production using a three cell model has been reviewed by Feldmann and Nossal (41).

VI Cell Mediated Immunity.

As has been discussed in the previous section, the activation of
B lymphocytes proceeds by a complex series of events probably involving,
for most antigens, the collaboration of T and B cells, and macrophages.
Like B lymphocytes, T cells bind antigen (101), demonstrate functional
specificity (9), and are effector cells. Is it likely then that T cell
activation is analagous to B cell activation?

Studies involving in vitro T cell function (M.I.F. production, ^{3}H-TdR
uptake, and cytotoxicity) have shown that:

(1) Upon activation by antigen, T lymphocytes differentiate to
become blast cells (126).

(2) In the presence of the sensitizing antigen, T cells secrete a
variety of non-specific factors (lymphokines) such as migration
inhibition factors, chemotactic factors, cytotoxic factors,
and mitogenic factors, which appear to play a role in cell-
mediated immunity ([10]).

(3) In the presence of the sensitizing antigen, T cells differentiate
into cytotoxic effector cells (50).

Hapten-carrier experiments on delayed hypersensitivity reactions
suggest that specificity in MIF production(25), lymphocyte proliferation
(84), and antigen induced cytolysis (50), is carrier dependent. These
data, obtained with structurally undefined DNP-protein conjugates have
led to the almost dogmatic view that cells reacting in cell-mediated
responses bear a receptor with a complex specificity pattern, containing
elements of both hapten and carrier (115), that there is a requirement for

a larger functional antigenic determinant than that required for inter-
action with B cells or circulating antibody (42) and that the recognition
is not as specific as in humoral immunity. Many results obtained using
defined low molecular weight antigens are in direct contradiction to the
studies involving complex undefined hapten-carrier systems. The mono-
valent molecule azobenzenearsonate-L-tyrosine (ABA-Tyr) demonstrates
positive skin tests (62,81,82), and stimulates lymphocytes into cell
division in vitro (4,5,16). The carboxy-terminal dodecapeptide of Bovine
glucagon also stimulates in vitro cell division in sensitized animals.

Several workers suggest that T cell recognition, although extremely
sensitive, may be less specific than that demonstrated in antibody pro-
duction:

(1) Hoffman and Kappler (53) and Playfair (91) and Marshall-Clarke demonstrated cross-
reactivity of helper T cells to erythrocyte antigens which
exhibited no serological cross-reactivity.

(2) Thompson et al. (123) described cross reactions between lysozyme
and carboxymethylated lysozyme in cell mediated immunity.
Previously, Gerwing and Thompson showed no serological cross-
reactivity between lysozyme and its carboxymethylated derivative
(43).

(3) Cross-reactivity in cell mediated immunity was shown by Parish
(86) using flagellin and acetoacetylated flagellin in rats and
in mice by Cooper and Ada (22) using flagella from two strains of
Salmonella that show no serological cross-reactivity.

Several possibilities exist to explain these observations:

(1) Differences between T and B cells are a reflection of sensitivity although the receptors may be the same. A lower affinity requirement for the stimulation of T cells is suggested by Parish (86).

(2) Apparent differences in sensitivity of T and B cells may reflect the difference in sensitivity of detection of function although they possess identical requirements for stimulation.

(3) There are determinants that are specific for T cells and stimulate T cells but not B cells. In the case of cross-reactions, it would be assumed that common T cell determinants were present.

This last possibility, that there is a functional dissociation of determinants for T and B cells is supported by the finding that certain antigens induce cell-mediated reactions rather than humoral responses (66,126). Further evidence has been found using synthetic and natural antigens:

(1) Senyk et al., using the natural polypeptide glucagon, found antibody specificity primarily for the amino-terminal heptadecapeptide, while only intact glucagon and its carboxy-terminal dodecapeptide stimulated cell division in sensitized cells in culture (112,113).

(2) Using the synthetic conjugates ABA-poly-D-glutamic acid and ABA-DNP, Alkan et al. (4) found that cellular immunity was directed against the ABA group (carrier) while antibody was produced to the DNP and PDG (hapten).

It is interesting, however, that when ABA is joined to a protein moiety its role is reversed and it acts as a hapten (108). The C-terminal undecapeptide of glucagon acts in an analogous manner (113).

Work with haptenic peptides from tobacco mosaic virus protein (118) and from oxidized ferredoxin (135) has shown that:

> There is no direct correlation between delayed skin reactions and lymphocyte stimulation. Haptenic peptides, when not bound to a carrier, elicit skin reactions and MIF in immune animals but do not stimulate thymidine uptake by lymphocytes. Hence, carrier specificity cannot be a requirement for the production of MIF. This is contrary to David and Schlossman (27) who showed that only immunogenic DNP-oligolysines elicited MIF production.

From these observations, it appears that for T cells, activation may occur without subsequent proliferation:

(1) Bloom (10) found that production of antigen induced MIF is dissociated from DNA synthesis and is associated with a non-dividing cell.

(2) Rocklin (96) using Budr-light inactivation of dividing cells, found production of MIF was unaffected by removal of lymphocyte proliferation in response to antigen.

These data suggest that either one population of T cells responds differentially to different antigenic stimulus or that two functionally separate populations exist.

VII   Cell Cooperation in Cell Mediated Responses

Lonai and Feldman (65,66) reported synergism between T and B cells in in vitro xenograft reaction, implicating lymphocyte interactions in cell mediated immunity. However, using column purified T cells, Goldstein et al. demonstrated complete autonomy of T·cells for sensitization (45) and cytotoxic effector function (46). The possibility that T-T interaction occurs is supported by several lines of investigation:

(1) Raff and Cantor (94) have demonstrated the presence of T cell subpopulations in the mouse.

(2) T-T synergism in GVH reactions has been claimed by Asofsky (6) and Tigelaar and Asofsky (124).

(3) The results of Schendel et al. (105) that mixed leukocyte culture (MLC) activation is required for the cell mediated lymphocysis (CML) reaction and that the cells responding in MLC are separable from those mediating CML by adsorption on specific monolayers (7) imply that cell-cell cooperation is involved in the CML reaction.

There is no direct evidence for either T-B or T-T interaction in cell-mediated immunity to soluble protein antigens. A two cell collaboration is suggested, however, by the work of Waterfield et al. (135) and Levy et al. (64) using chemically defined peptides:

(1) Only divalent or polyvalent antigens (more than one antigenic determinant per molecule) are immunogenic.

(2) Only immunogenic molecules stimulate thymidine incorporation in lymphocytes from sensitized animals. Haptenic peptides do not. In some situations however, molecules containing a single antigenic determinant to which the cells are sensitized are sufficient for stimulation of thymidine uptake.

These results suggest some sort of bridging mechanism for T cell activation and the work in this thesis was designed to further test this hypothesis.

Mitchell and
The antigens synthesized by Levy et al. contain the amino-terminal heptapeptide and the carboxy-terminal pentapeptide which have been identified as the antigenic determinants of the ferredoxin molecule (73). These peptides can be synthetically linked to form immunogenic analogs of the natural protein. For the major part of this investigation, the peptide $N_{10}C$ (a synthetic analogue of oxidized ferredoxin from C. pasteurianum) has been used since the two chemically distinct antigenic determinants would be postulated to react with at least two different populations of lymphocytes. Cell cooperative models proposed for the production of humoral antibodies postulate the existence of different lymphocyte populations which interact with separate antigenic determinants on the eliciting antigen. If an analogous situation exists in cell-mediated immunity, then the divalent ferredoxin peptides can be used to look at cell interaction in delayed hypersensitivity reactions using the following approach:

(1) Removal or inactivation of cells reacting with either antigenic determinant should abrogate responses requiring cell cooperation.

(2) Mixing a cell population functionally depleted in cells reacting to only one antigenic determinant with one depleted in cells reacting to the other determinant should restore cell cooperative responses.

Specific inactivation of cells binding a single antigenic deter-
minant was attempted using radioactive antigen suicide in this thesis.

VIII "Antigen Suicide"

Radiolabelled antigen binding cells were first examined for immuno-
competence by Ada and Byrt using an "antigen-suicide" technique (3).
Spleen cells from unimmunized mice were incubated with polymerised flag-
ellin labelled to high specific activity with ^{125}Iodine. After removal
of unbound antigen, treated cells were stored overnight to allow radia-
tion death of cells binding antigen. Cell populations pretreated with
radiolabelled flagellin failed to react to a subsequent challenge with
the antigen. Response to unrelated antigens remained intact, demonstra-
ting specific clonal deletion.

It has long been known that lymphocytes display marked sensitivity
to ionizing radiations. With this in mind, Ada and Byrt explained their
results in the following manner: Antigen binding cells specific for ^{125}I
labelled flagellin adsorbed the antigen onto their surface. During iso-
tope decay, electrons of relatively weak energy are emitted and are
absorbed a few microns from their source. Cells binding significant
amounts of antigen receive relatively high doses of ionizing radiation
and are inactivated. Other cells are left unharmed. Evidence supporting
specific clonal elimination of antigen binding lymphocytes by antigen
suicide is derived from several sources:

    (1) Specific removal of antibody response to hemocyanin in both
        immune and non-immune animals was achieved by Unanue (132)

using ^{125}I hemocyanin suicide <u>in</u> <u>vitro</u>.

(2) Humphrey and Keller (55) used the synthetic antigen ^{125}I-
TIGAL <u>in</u> <u>vivo</u> and selectively inhibited the response to that
antigen.

Antigen suicide techniques are adaptable to studying hapten-carrier
function and cell cooperation in the immune response:

(1) Using an adoptive transfer system, Basten <u>et</u> <u>al</u> showed that
specificity of the antibody response to fowl gamma globulin
and horse erythrocytes was dictated by both T and B lymphocytes
(9).

(2) Roelants and Askonas were able to test helper cell function in
an adoptive transfer system using DNP-ovalbumin and DNP-
hemocyanin. They specifically removed helper function for
anti-DNP response using high specific activity, ^{125}I-hemocyanin
(100).

(3) Specific inactivation of DNP hapten-binding cells using irradi-
ation suicide was reported recently by Golan and Borel (44).

On a theoretical basis, specific antigen suicide has the potential
to functionally impair cells binding a single antigenic determinant and
thus may be one of the most powerful techniques for investigating aspects
of molecular recognition and cell-cooperation in a functionally hetero-
genous lymphocyte population.

IX   Thesis Objectives.

(1)   To develop a microculture system for measuring mitogen and antigen
      induced stimulation of uptake of radioactive isotopes by lymphocytes.

(2)   To examine antigen binding cells for ferredoxin peptides using auto-
      radiography and cell separation techniques.

(3)   To develop an "antigen suicide" technique to specifically inactivate
      cells reacting with a single antigenic determinant.

(4)   To test for cell-cell interaction in the cell-mediated immune res-
      ponse to ferredoxin peptides using functional inactivation of cell
      populations by "antigen suicide" techniques.

MATERIALS AND METHODS

I   Peptide Synthesis

In the solid phase method of peptide synthesis, the peptide being synthesized is covalently attached to an insoluble copolystyrene-divinyl benzene resin and this is insolubilized or in "solid phase". Reagents used in the synthesis, along with unattached amino acids remain soluble and can easily be washed from the resin. The necessity for purification procedures at intermediate steps in the synthesis is thus eliminated. The reaction sequence is as follows: (Figure 1).

(1) Chloromethyl groups on the resin react with the salt of a t-butyloxycarbonyl (t-Boc) amino acid to form an ester linkage with the carboxyl of the first amino acid.

(2) The t-Boc group protecting the amino group of the attached amino acid is removed with anhydrous trifluoracetic acid to allow it to react with the carboxyl group of the incoming protected amino acid.

(3) The coupling reaction is carried out by dicyclohexylcarbodiimide and involves formation of a peptide bond between the amino group of the coupled amino acid and the carboxyl group of the incoming amino acid, its amino group being protected by t-Boc.

The deprotection, addition of new protected amino acid, and coupling steps are repeated until the peptide sequence is complete.

(4) The completed peptide is cleaved from the resin by a nucleophilic displacement reaction using HBr gas. The terminal t-Boc group is removed at the same time by an elimination reaction.

Figure 1. Flow Diagram of Solid Phase Peptide Synthesis

(1)

Anhydrous
Trifluoracetic Acid

DEPROTECT : NEUTRALIZE

(2)

isobutylene   amino acyl polymer

t-BOC amino acid diimide

COUPLE

(3)

t-BOC peptide polymer

HBr

CLEAVE

(4)
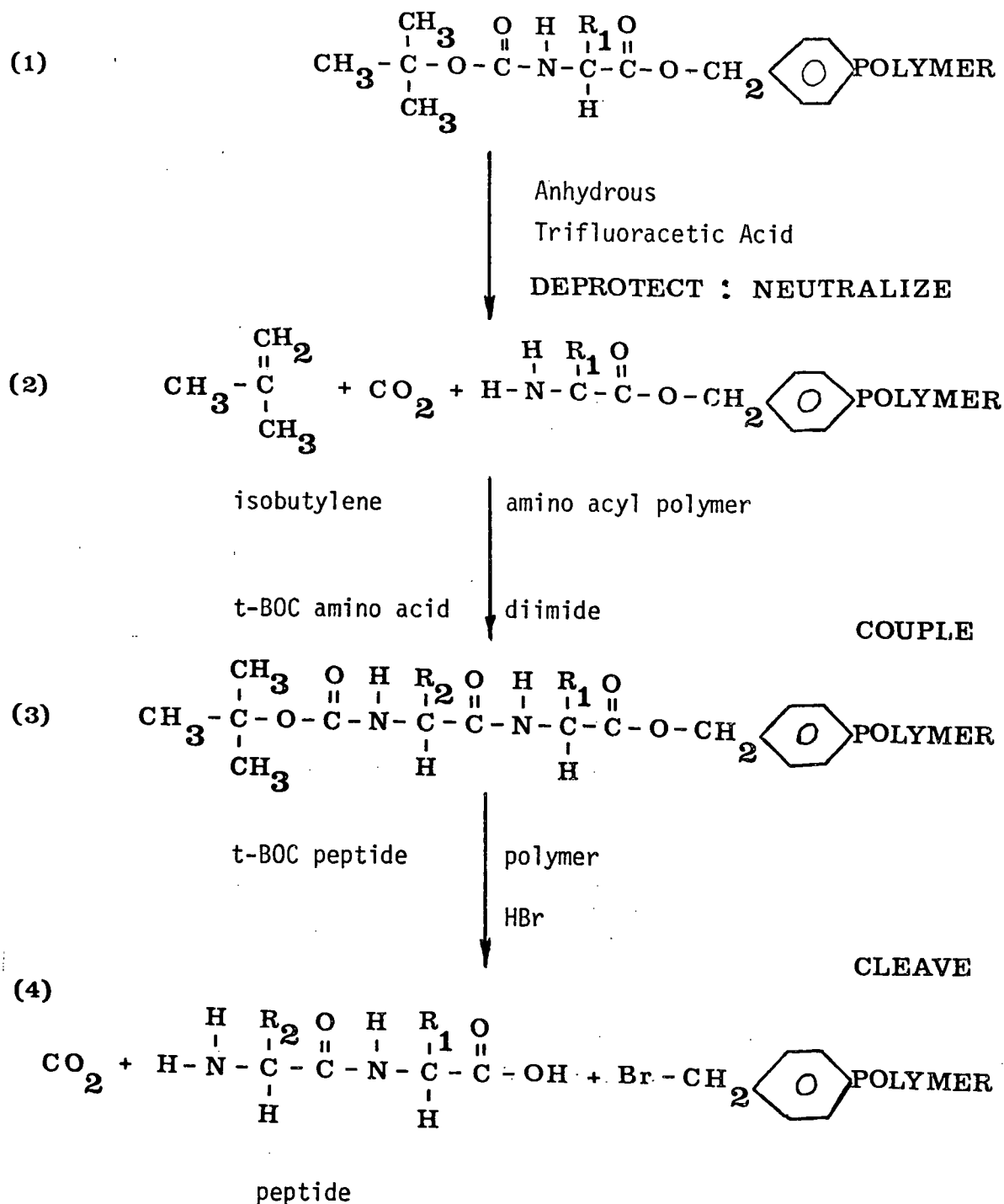
peptide

The following peptides, synthesized by the solid phase method of Merrifield (71) with the modifications suggested by Stewart and Young (119), were used:

(1) NH_2-terminal haptenic heptapeptide from the oxidized ferredoxin molecule of <u>Clostridium</u> <u>pasteurianum</u> (Figure 2).

<u>Sequence</u>: NH_2-Ala-Tyr-Lys-Ile-Ala-Asp-Ser-COOH

<u>Designated</u>: N

(2) COOH-terminal haptenic pentapeptide from the same molecule (Figure 2).

<u>Sequence</u>: NH_2-Ala-Pro-Val-Gln-Glu-COOH

<u>Designated</u>: C

(3) The two haptenic peptides bridged by ten amino acids (Figure 2).

<u>Sequence</u>: NH_2-Ala-Tyr-Lys-Ile-Ala-Asp-Ser-$(Gly)_{10}$-Ala-Pro-Val-Gln-Glu-COOH

<u>Designated</u>: $N_{10}C$

(4) A symmetrical molecule consisting of two carboxyterminal pentapeptides bridged by one malonic acid and ten glycine residues.

<u>Sequence</u>: COOH-Glu-Gln-Val-Pro-Ala-Mal-$(Gly)_{10}$-Ala-Pro-Val-Gln-Glu-COOH

<u>Designated</u>: $C_{mal}C$

(A space-filling model of $N_{10}C$ is shown in Figure 3.)

Figure 2. Amino acid sequence and haptenic determinants of the synthetic immunogen (N-10-C).

NH_2-Ala-Tyr-Lys-Ile-Ala-Asp-Ser-(Gly_{10})-Ala-Pro-Val-Gln-Glu-COOH

Figure
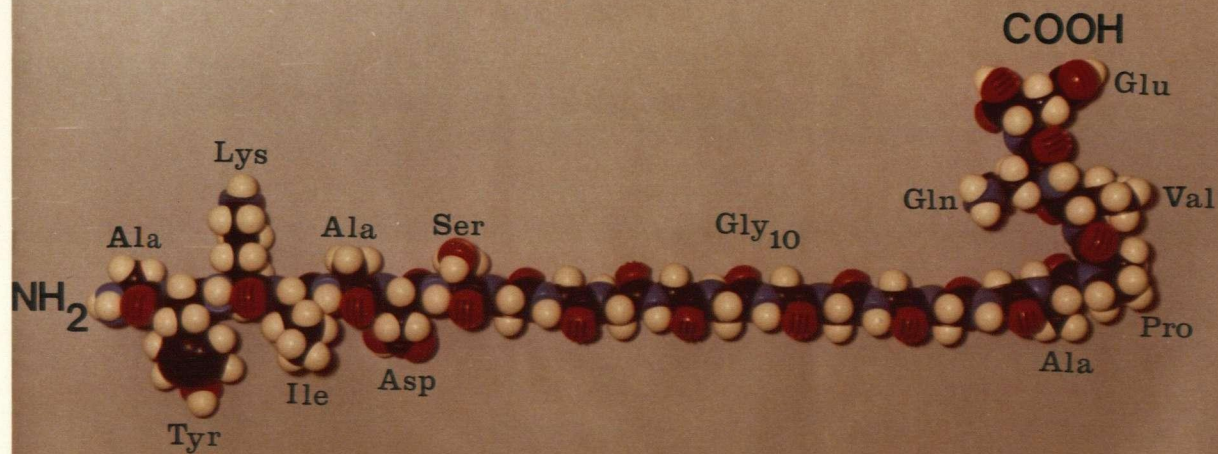
Figure 3.   Space-Filling Model of the Synthetic Immunogen $N_{10}C$.

An automated synthesizer built by Mr. Doug Hull in Dr. Levy's laboratory was used in all syntheses. T-Boc amino acids were purchased from Sigma Chemical Company, or from Schwarz-Mann, and the chloromethylated resin from Bio-Rad. Peptides were cleaved from the resin using HBr. Tyrosine containing peptides were cleaved with HBr scrubbed with anisole. Peptides were purified by gel filtration on Sephadex G-15 and G-25 columns. After acid hydrolysis, representative aliquots were analyzed for amino acid content on a Beckman Model 120 amino acid analyzer. Expected and observed ratios of amino acids of the synthesized peptides are presented in Table I. Synthesis and purification from Levy et al (64).

II Performic Acid Oxidation of Ferredoxin.

Ferredoxin from Clostridium pasteurianum was purchased from Sigma Chemical Co. (F3250 Type 1), and its performic acid oxidized derivative (OFD) was prepared by the method of Tanaka et al (121) in the following manner: 30 mg Ferredoxin was dissolved in 4.0 ml distilled H_2O and 2.0 ml 15% TCA + 0.1 ml 2-mercaptoethanol added. The mixture was allowed to stand for 1 hour at 4 C. The precipitate was collected by centrifugation and washed consecutively with 5.0 ml dH_2O, 5 ml 95% ethanol and 5.0 ml ether. The final precipitate was dried under vacuum, dissolved in 2.0 ml concentrated formic acid and 4.0 ml performic acid (prepared from 9.0 ml formic acid + 1.0 ml 30% H_2O_2) was added. The oxidation was allowed to proceed for 4 hours at -10 C. The oxidized ferredoxin was lyophilized, dissolved in 10.0 ml distilled H_2O and quantitated by amino acid analysis.

Table I. The amino acid composition and expected molar ratios of the amino acids in the synthetic peptides used in this study. Peptide sequences are given on page 25.

| Amino acid | Amino acid molar ratios of synthetic peptides (M/M) | | | | | | | |
| | N[1] | | C[2] | | N10C[3] | | CmalC[4] | |
	Expected	Found	Expected	Found	Expected	Found	Expected	Found
Aspartic	1.0	1.16			1.0	0.91		
Serine	1.0	0.94			1.0	1.02		
Glutamic			2.0	2.02	2.0	2.19	4.0	4.30
Proline			1.0	1.03	1.0	1.40	2.0	2.20
Glycine					10.0	10.26	10.0	9.50
Alanine	2.0	2.05	1.0	1.00	3.0	3.30	2.0	1.90
Valine			1.0	0.94	1.0	1.20	2.0	2.07
Isoleucine	1.0	0.98			1.0	0.36		
Tyrosine	1.0	0.85			1.0	0.76		
Lysine	1.0	1.50			1.0	0.94		

[1] Synthesized by T. Pearson [3] Synthesized by D. Waterfield

[2] Synthesized by D. Hull [4] Synthesized by D. Hull

III Immunization Procedures.

Random bred male guinea pigs weighing 500-800 g were immunized with $N_{10}C$ or OFD in sterile saline and 50% by volume of complete Freund's adjuvant (Difco) in a total volume of 1.0 ml. Each guinea pig received 250 µg total, in the following manner: 0.2 ml intramuscularly into the shoulder muscles and 0.2 ml intraperitoneally. Guinea pigs were immunized with $C_{mal}C$ in an identical manner. In several experiments, animals were immunized with 250 µg keyhole limpet hemocyanin (KLH) mixed with the OFD dose. All animals were skin tested 11-14 days after initial sensitization.

IV Skin Tests.

Hair was removed from the flanks of the test animals by shaving followed by treatment with a depillating agent. Test antigens (50 - 100 µg in 0.1 ml sterile saline) were injected intradermally along with a saline control. Results were read at 3 hours (immediate reaction) and 24 hours (delayed reaction) from the time of challenge. Erythema of greater than 0.6 cm was considered positive, as saline controls were always less than 0.6 cm in diameter.

V Tissue Culture Medium.

Guinea pig cells were cultured in R.P.M.I. 1640 (Gibco) containing 15% heat inactivated ($56^{o}C$, 30 min) fetal calf serum, 100 units penicillin and 100 µg streptomycin per ml. Medium components were made up separately and combined after sterilization by millipore

filtration. The pH was then adjusted to 7.1 - 7.2 with 1N HCl. This medium is termed complete medium. Incomplete medium contained no fetal calf serum.

VI Preparation of Cells.

Guinea pigs were sacrificed by a sharp blow on the head and were exsanguinated by heart bleeding. The animals were shaved and soaked with 70% ethanol. Mesenteric, inguinal, popliteal and axillary lymph nodes were removed aseptically, trimmed of fat, and teased gently apart in phosphate buffered saline, containing 5% FCS, using curved forceps. Cell clumps were broken up by expulsion through a tuberculin syringe with a number 26 needle. The cells were washed once by centrifugation, resuspended in 1.0 ml PBS and centrifuged through a discontinuous fetal calf serum gradient (100%, 50%, 25% FCS in PBS) to remove debris. Spleen cell suspensions were prepared in an identical manner. Viable cell numbers were determined by trypan blue exclusion. All procedures were carried out at room temperature in a laminar flow air hood.

VII Migration Inhibition.

Migration inhibition was performed as a modification of the method described by David et al (26). Capillary tubes were filled with a 20% spleen cell suspension and plugged with modelling clay. They were centrifuged at 170 x g for 5 minutes, cut just below the cell-fluid interface and placed on the bottom of Mackaness type

chambers. Measurement of migration in the presence and absence of antigen was performed by determining the rectangular area encompassing the furthest extent of confluent macrophage travel, using a calibrated microscope stage. All tests were performed in duplicate and two capillaries used in each test.

VIII Lymphocyte Culture.

A miniaturized system of lymphocyte culture and stimulation was developed. The method is derived from the mixed leukocyte culture (MLC) test of Hartzman et al. (48). Lymph node cells in a total volume of 0.25 ml were placed in the wells of flat bottomed microtitre plates (LINBRO ISFB 96-TC) covered with sterile plastic lids (LINBRO #55), and incubated in a CO_2 enriched humidified atmosphere. Cell concentration, antigen and mitogen concentration, and length of incubation were varied in order to optimize culture conditions. 1 microcurie ^{3}H-thymidine (specific activity 2 Ci per mmole-NEN) was added to each microtitre culture well 16 - 18 hours prior to harvesting. All materials were dispensed using automatic repeating syringe dispensers (Hamilton Co. #PB-600-1). Triplicate cultures were run in most experiments, quadruplicate or sextaplate in others.

IX Harvesting of Radioactively Labelled Cultures.

Lymphocyte stimulation was studied using the incorporation of tritiated thymidine into DNA. Due to the technical burden of harvesting multiple samples and the length of time required to precipitate

each microtitre culture separately, a machine which performs simultaneous precipitation of several microtitre cultures was built. The apparatus was re-designed from the prototype observed by the author in Dr. Fritz Bach's laboratory in Madison, Wisconsin. A similar model is discussed by Hartzman et al. (49).

Harvesting of lymphocyte microcultures was achieved quickly and efficiently using a machine built in this department. After appropriate incubation of lymphocyte microcultures, tritiated thymidine is added to the culture and 16 hours later the well contents are aspirated onto glass fibre filters. The filters are then washed, placed in glass vials and dried in an oven at 100 C for 30 minutes. Scintillation fluid was added (60% toluene, 40% methanol, and 41 ml NEN Liquifluor in 1.0 liter) and the samples counted for one minute on a Nuclear Chicago scintillation counter (Model 725). Using the microprecipitator apparatus, one row of microtitre plate wells (12 cultures) can be harvested in about one minute.

The microprecipitator consists of four main parts: (1) a reservoir-valve control unit, (2) an aspirator-washer unit, (3) a filter unit, (4) a vacuum unit (Figure 4).

The reservoir-valve control unit consists of a glass bottle reservoir (containing saline or distilled water) connected to the washer unit by tygon tubing. The flow of wash fluid from the reservoir is controlled by a microswitch activated solenoid valve. Flow is by gravity feed. It is possible to add TCA and methanol washes by the addition of

two extra microswitch-solenoid combinations.

The aspirator-washer unit (Figure 5,6) functions to remove the cultures from the microtitre wells, to wash the wells free of cells and to aspirate the cultures and washings onto glass fibre filters where they are washed free of unincorporated isotope. The tubing from the reservoir allows the appropriate wash fluid to flow into the hollow acrylic handle. The fluid flows down the outside of 12 pieces of stainless steel tubing which project into the microtitre wells. Microtitre plates are fixed in place by a spring-loaded acrylic wedge attached to the base plate. The stainless steel tubes are connected to 12 lengths of tubing which carry the well contents and wash fluid to the wells in the filter unit.

The filter unit (Figure 5, 7) holds the glass fibre filter strips (Reeve Angel) to allow application of suction and removal of filtrate. An acrylic baseplate contains 12 porous polypropylene discs upon which the filter strips are placed. The 12 polyethylene tubes from the aspirator-washer unit fit into 12 holes in the top piece of the filter unit. A filter strip is placed on the base plate and the top piece is clamped firmly down using a manual quick-release clamp. An "o" ring at the bottom of each top-piece cylinder prevents vacuum leakage, leakage from one aspirating channel to the next and also "punches out" glass fibre discs from the filter strip.

The vacuum unit consists of an electric vacuum pump connected via two liquid traps to an exhaust port on the base of the filter unit. All

filtrates are collected in the traps and are disposed of as radioactive waste.

A normal operating sequence is as follows: The top unit is clamped in place over a filter strip and the vacuum turned on. The contents of the microtitre wells are aspirated onto the filters and the wells washed with water by activating the solenoid, allowing water to flow into the wells. The wells are scraped free of adherent cells with the plastic tube tips. After 15 - 20 seconds of constant washing, the clamp is released, the filter peeled back and the filter discs remaining are removed after turning off the vacuum. Originally, cells were washed with saline, TCA and then methanol. Washing with water gives identical results.

The aspirator and filter units can be easily dissassembled and background radioactivity removed by overnight soaking in "Count off" fluid (NEN). Background counts are usually in the order of 10 - 20 cpm/filter disc. Providing there is adequate washing, no contamination is found from one filter strip to the next.

X   Preparation of Succinylated BSA (s-BSA).

Bovine serum albumin (200 mg, twice crystallized, Miles Research Laboratories, Kankakee, Ill.) in 3.0 ml distilled water, was placed in pencil dialysis tubing (Visking, Union Carbide) and dialyzed exhaustively against 0.1 N NaCl to remove contaminating ions. The pH was adjusted to 7.5 with N NaOH and 500 mg succinic anhydride added gradually with stirring over a one hour period. pH was maintained at 7.5 with 5.0 N NaOH. Stirring was continued for one more hour and reactants then removed by exhaustive dialysis at 4 C against distilled water.
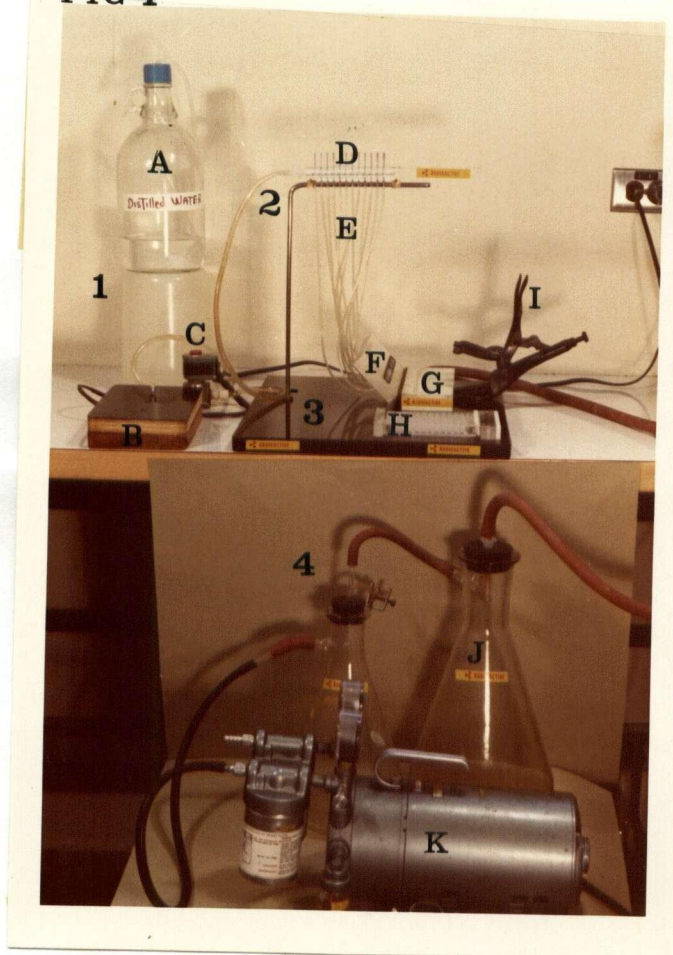
Figure 4. Microharvester Apparatus.

1. <u>Reservoir-Valve Control Unit</u>

    a) Reservoir
    b) Solenoid Valve Microswitch
    c) Solenoid Valve

2. <u>Aspirator-Washer Unit</u>

    d) Aspirating Needles
    e) Tygon Tubing

3. <u>Filter Unit</u>

    f) Top Plate
    g) Filter Holder
    h) Microtitre Plate
    i) Clamp

4. <u>Vacuum Unit</u>

    j) Vacuum Traps
    k) Vacuum Pump

Figure 5.   The aspirator and filter units.

Micro Precipitator

Oblique Projection

HANDLE

.355 ±.0005

.084 Dia S.S. Needles

.600 ±.0002

LID

.032 ±.001

BASE
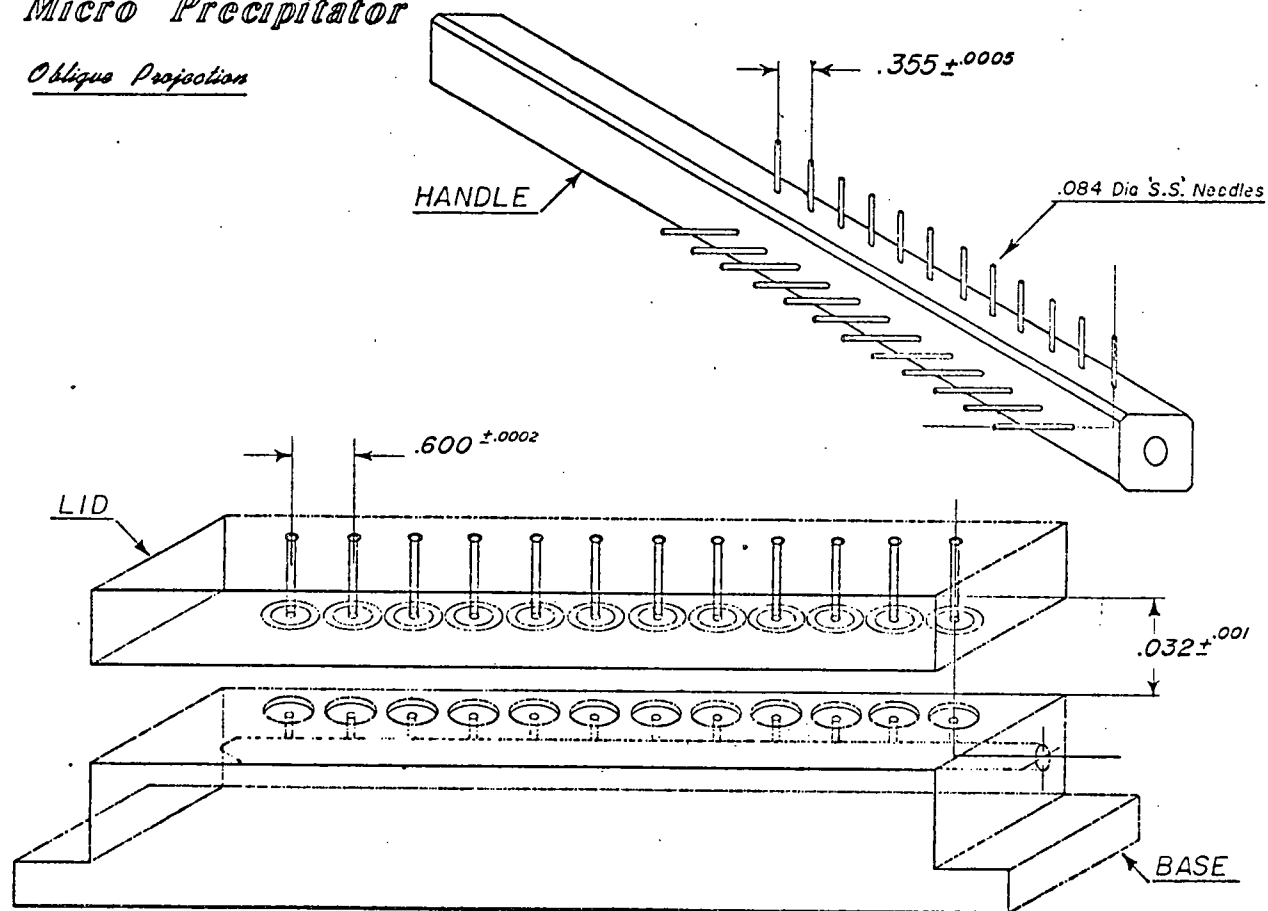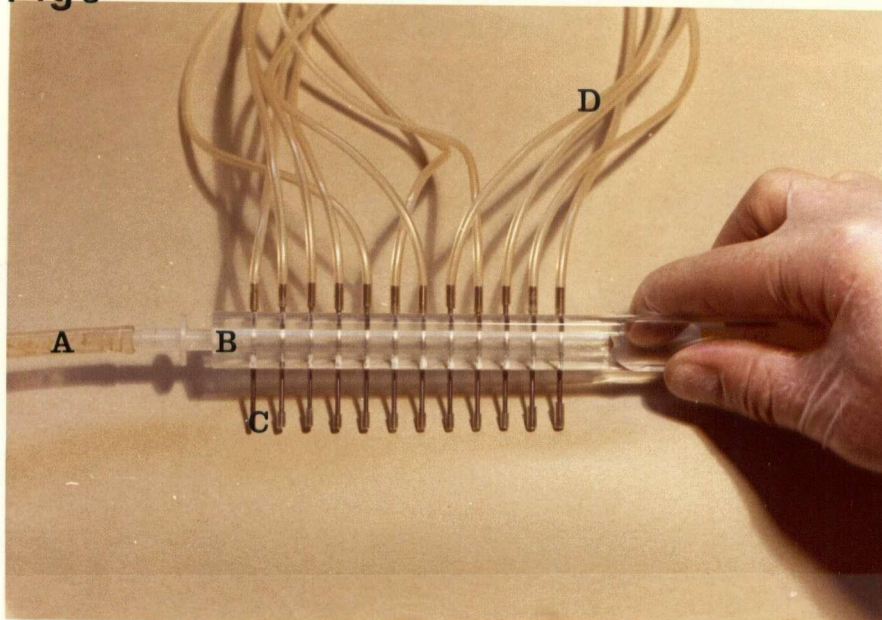
Figure 6 .   <u>The Aspirator-Washer Unit - Detail</u>

    a) Tygon tubing from Reservoir
    b) Acrylic handle
    c) Stainless steel aspirator needles - Tygon tipped
    d) Tygon tubing - to filter unit

Figure 7 .   <u>The Filter Unit - Detail showing Removal of Filter Discs</u>

    e) Base plate
    f) Top plate showing rubber "O" rings
    g) Filter holder
    h) Clamp
    i) Microtitre plate

XI Coupling of Peptides to s-BSA.

The NH_2-terminal heptapeptide and the COOH-terminal pentapeptide of the ferredoxin molecule were conjugated to s-BSA via a water soluble carbodiimide reaction. This procedure was based on the method for modification of carboxyl groups in proteins of Hoare and Koshland (51). 1 Ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (EDCI) was obtained from OTT Chemical Company. In a total volume of 1.0 ml of water, 10 mg of s-BSA and 10 mg of the peptide were mixed. The pH was adjusted to 4.75 with 0.1N HCl and 0.25 ml of DH_2O containing 100 mg EDCI was added. The reaction was allowed to proceed for 2 hours with constant stirring and the pH maintained at 4.75 with 1N HCl during this time. A further 100 mg EDCI was added to complete the reaction. Reactants were removed by dialysis at $4^{o}C$ against 0.001N HCl (4 x 1000 ml over 48 hr). The conjugates were solubilized by dropwise addition of 5N NaOH to pH 12.0. After 3 - 12 hr, the pH was adjusted to 7.0 with slow addition of 1N HCl. Succinylated BSA was used to ensure that coupling occurred exclusively through the free amino groups of the peptides.

XII Amino Acid Analysis.

Protein concentrations were determined by the method of Lowry et al (67), and approximately 0.02 μmoles of each conjugate acid hydrolyzed for analysis (6N HCl, 18 hr, $105^{o}C$ in vacuo). The hydrolysates were dried in a flash evaporator, washed free of acid with distilled water and quantitated by amino acid analysis on a Beckman

Model 120 amino acid analyzer by the method of Spackman et al (117).
The degree of coupling of peptides was estimated by calculating
differences in key amino acids between the conjugates and s-BSA;
these being indicative of the amount of peptide present in each con-
jugate.

XIII Iodination of Antigens.

Iodination was performed in microvolumes by a modification of
the procedure of Hunter and Greenwood (47). 0.02 Millicuries of
carrier free ^{125}Iodine as NaI (0.01 ml in 0.1 N NaOH, New England
Nuclear) was mixed with 2 - 10 µg protein (0.02 ml in 0.4 M phosphate
buffer, pH 7.5) and chloramine T (sodium salt of N-chloro-p-toluene
sulfonamide, Eastman Kodak - 200 µg in 0.02 ml) was added. Iodination
was allowed to proceed 1 - 5 minutes and the reaction then termina-
ted by addition of sodium metabisulfite (200 µg in 0.02 ml). Non-Radioactive
KI was then added (0.05 ml of 1% solution) and the reactants removed
by gel filtration on Sephadex G-15 or by dialysis against a continu-
ous flow of saline. All manipulations were carried out in a fume
hood and reagents added with micro pipettes inserted into specially
adapted tuberculin syringes. Cold iodinated (^{127}Iodine) antigens
used as controls, were prepared in an identical manner.

XIV Gel Filtration.

A 30 x 1 cm column was packed to a height of 10 cm with Sephadex
G-15. The column was equilibrated with 0.04 M phosphate buffer pH 7.5

and then treated with 5.0 ml heat inactivated fetal calf serum (FCS) in order to inhibit non-specific adsorption of protein. A drop of FCS was added to each sample prior to application to the column. Eluting buffer was 0.04 M phosphate pH 7.5 and the flow rate was adjusted to 12 drops per minute. Fractions of 8 drops per tube were collected by hand and tubes read using a Geiger-Muller counter (Nuclear Chicago). Peak tubes were pooled and adjusted to physiological osmolarity with NaCl.

XV   Determination of Specific Activity.

All iodinated peptides and proteins were quantitated after adjusting for loss on the column or on the dialysis membranes. Specific activity was calculated by converting CPM to DPM after counting on an Isocap 300 scintillation counter (Iodine channel is 80% efficient). Antigens were frozen and specific activity calculated on the basis of a 60.2 day half-life for the ^{125}I isotope.

XVI   Immunological Activity Testing

All synthesized peptides, s-BSA conjugates and their iodinated derivatives were tested for immunological function both in vivo by using skin tests and in vitro by migration inhibition.

Denaturation of proteins can occur in the presence of chloramine-T used in Iodination procedures (85). Iodinated antigens were thus tested for activity by skin testing with ^{127}I labelled proteins and assuming that analogous ^{125}I labelled proteins would react in an identical manner.

XVII <u>Antigen Suicide.</u>

Radioactive "antigen suicide" was carried out as in Figure 8,
and is a modification of the procedure of Ada et al. (3). Guinea pig
spleen or lymph node cells were incubated with antigens labelled to a
high specific activity with ^{125}I. Cells (2 x 10^7 in 0.5 ml complete
1640 + 0.015 M sodium azide) were placed in 10 ml disposable plastic
test tubes (Fisher #2001) and cooled to 4^oC. Antigens (100 ng - 2
µg in 0.2 ml saline) were cooled to 4^oC and mixed with the cells.
The total volume was made up to 2.0 ml with complete 1640 + azide
medium and the mixture was incubated for 1 hour at 4^oC. Incubation
was at 4^oC in the presence of azide in order to inhibit uptake of anti-
gen by macrophages. The cells were then centrifuged from the mixture
(750 x <u>g</u>, 5 minutes), the supernate removed by aspiration, and the
cells resuspended in the same medium containing azide (1.0 ml). Un-
bound antigen was removed by centrifuging the cells through three 1.0
ml discontinuous fetal calf serum gradients (100%, 75%, 50%, 25% in
PBS). The cell pellet was then resuspended in 0.2 ml PBS + 15% FCS
and stored at 4^oC for 24 hours to allow radiation death. Cells were
resuspended in 1.0 ml PBS and centrifuged through a discontinuous fetal
calf serum gradient to remove unbound antigen and cell debris. The
washed cells were resuspended in complete 1640 medium, incubated at
37^oC for 2-3 hours, counted and prepared for lymphocyte stimulation,
MIF tests and autoradiography. Control cells were treated in an iden-
tical fashion but with saline or ^{127}I labelled antigens.

Figure 8.   <u>Antigen Suicide Procedure - Flow Chart</u>

Lymph node cells (spleen cells)

+

^{125}I Antigens

^{127}I Antigens (Control)

↓ 4°C 1 hour

Centrifuge through FCS gradients 3x

↓

Resuspend cells in 0.5 ml PBS + 15% FCS

Refrigerate at 4°C 24 hours to allow irradiation death

↓

Incubate at 37°C 2 - 3 hours

↓

Centrifuge through FCS gradients

↓

Resuspend in medium, count viable cells

Lymphocyte stimulation   Autoradiography   MIF (spleen cells)

XVIII   Antigen Binding Studies.

Varying concentrations of iodinated antigens were incubated with 2 x 10^7 guinea pig lymph node cells in a total volume of 0.2 ml in complete medium 1640 + 0.015 M sodium azide at 4 C for 1 hour. The cells were then freed of unbound antigen by centrifugation through FCS gradients (X3), resuspended in 0.2 ml complete medium 1640, precipitated with 15% TCA (0.8 ml) and triplicate samples pipetted onto glass fibre filters. The filters were then washed with PBS and methanol, dried and counted in an Isocap 300 scintillation counter. A range of antigen concentration was tested in order to ascertain saturation curves for use in antigen suicide experiments.

XIX   Antigens for Binding Experiments and Autoradiography.

Proteins were labelled to low specific activity with ^{125}Iodine by a chloramine-T method modified from Hunter and Greenwood (47). One mCi ^{125}Iodine as NaI (N.E.N. - NEZ-033) was mixed with 100 μg protein (0.1 ml in 0.4 M phosphate buffer pH 7.5). Chloramine T was added (0.02 ml of 20 μg/ml in phosphate buffer pH 7.5) and after one minute the reaction was terminated with the addition of 0.02 ml sodium metabisulfite (200 μg/ml in 4 M phosphate buffer, pH 7.5). Non radioactive KI was then added (0.05 Ml of 1% solution), the volume made up to 0.3 ml with FCS and the reactants removed by gel filtration on Sephadex G-15. Specific activities ranged between 0.1 and 5.0 μCi/μg protein.

XX Preparation of Gelatin-Coated Slides.

Glass slides were washed in chromic acid for 1 hour, rinsed in tap water 10 minutes and washed with several liters of distilled water. The slides were then immersed for 2 minutes into a gelatin solution pre-cooled to $21^{\circ}C$ (3.75 gm bacteriological gelatin in 750 ml distilled water). After dipping, excess gelatin was drained off and slides were dried at $37^{\circ}C$ for 2 hours. Slides were stored at $4^{\circ}C$ in sealed black boxes and discarded if more than 4 weeks old.

XXI Autoradiography.

Immune or non-immune lymph node cells were layered onto 100% FCS and clumps allowed to settle. The uppermost layer of cells was centrifuged and resuspended in complete 1640 + 0.015 M Sodium azide. The cells (1×10^7 in 0.2 ml) were cooled to $4^{\circ}C$ and then 1 µg ^{125}I antigen was added. The total volume was made up to 0.5 ml and the mixture kept at $4^{\circ}C$ for 60 minutes with periodic shaking.

Unbound antigen was removed by centrifugation of the mixture through five discontinuous FCS gradients. Cells were resuspended in 0.2 ml 50% FCS (in PBS) and painted onto gelatin coated slides using a fine paint brush. The slides were then fixed in methanol for 30 minutes, washed in water, dried, and dipped in Nuclear Track emulsion (Kodak NTB2). The slides were dried for 1 hour in a vertical position and stored for three days - one week at $4^{\circ}C$ in sealed black boxes prior to developing.

Autoradiography using high specific activity antigens was per-
formed directly on cells taken from antigen suicided cell preparations.

XXII Processing of Slides.

Autoradiograph slides were developed by immersion into DEKTOL
developer for 2 minutes, distilled water for 30 seconds and Kodak
fixer for 5 minutes. Softening of the emulsion was prevented by pre-
cooling all solutions to 17°C. Developed slides were washed in cold
running water for 30 minutes, air dried, and stained for 5 minutes
with 2% Giemsa in N phosphate buffer pH 7.0.

XXIII Examination of Slides.

Cells were examined under oil immersion using a light microscope.
Labelled cells were scored positive if they had a definite lymphoid
morphology and greater than 10 grains overlying them or immediately
adjacent. Between 10,000 and 20,000 cells were examined in each experi-
ment when using low specific activity antigens and 5,000 cells when
using high specific activity preparations.

XXIV Macrophage Autoradiography.

Peritoneal exudate cells were collected by washing the peritoneal
cavity of guinea pigs with sterile PBS sixteen hours after intraperi-
toneal injection of 20.0 ml of sterile mineral oil. The cells in the
washings were packed by centrifugation at 200 x $\underline{g}$ for 10 minutes and
then washed 2x with PBS. The cells were resuspended in RPMI 1640

complete medium and adjusted to 1.5 x 10^7 viable macrophages/ml. 3 x 10^6 cells were incubated with iodinated antigens (1 µg) in a total volume of 0.3 ml. Cells were freed of unbound antigen by centrifugation through three FCS gradients and were then smeared on gelatin coated slides and processed for autoradiography as previously described. Cells were scored positive if they had a definite macrophage morphology and greater than 10 grains overlying them. One thousand cells were counted in each experiment.

XXV Cell Separation-Nylon Columns

Guinea pig lymph node cell populations were fractionated by their differential adherence to nylon fibres in the following manner: Nylon fibre inserts were removed from LP-1 Leuko-Pak leukocyte filters (Fenwal Laboratories - Morton Grove, Illinois) and were soaked in changes of distilled water for 1 week at 4°C and for 24 hours at 37°C. The nylon was dried in an oven at 100°C for 2 hours and 0.6 g of dry fibre was packed into 5.0 ml syringe barrels fitted with poly propylene disc supports. The nylon 'columns' were sterilized by autoclaving. The columns were washed with complete 1640 medium (care being taken to remove all air bubbles) then filled with complete medium and incubated for 30 minutes at 37°C. The medium was run to the level of the nylon fibre and 5 x 10^7 - 1 x 10^8 cells in 1.0 ml complete 1640 were gently layered on with a Pasteur pipette. The cells were run into the column and washed in with 1.0 ml complete medium. The column

was topped up with 2.0 ml medium and incubated for 30 minutes at 37 C

in a CO_2 enriched, humidified atmosphere. Cells were eluted at 1 drop

per second, with the addition of medium until the effluent became clear

(approximately 5.0 mls). Viable cell numbers were determined by trypan

blue exclusion and aliquots then prepared for lymphocyte stimulation,

antigen suicide experiments and autoradiography. From Eisen et al (38).

XXVI Cell Separation - Anti IgG Plates

Anti IgG plate separation of cells was performed in collaboration

with Fumio Takei using a method developed by Mr. Takei in this labora-

tory. Essentially, the following procedure was followed: Sheep or

rabbit anti guinea pig IgG was purified by specific immunoadsorbance to

Sepharose 4 B beads coupled with DEAE purified guinea pig IgG via cyano-

gen bromide activation.

Ten gauge nylon strip (Polypenco 4") was cut to fit the bottom of

120 x 30 mm polystyrene petri dishes (Falcon). The nylon discs were

partially hydrolyzed (3.0 N HCl/37 C/2hr), washed with distilled water

and then glued onto the bottom of the petri plates with polystyrene

solution in toluene. The plate bottoms were washed with distilled

water and 5.0 ml of 2.5% glutaraldehyde in 0.2 M sodium bicarbonate

buffer (pH 9.4) was added to each plate and incubation at room tempera-

ture allowed for 30 minutes. The plates were washed with distilled

water to remove unattached glutaraldehyde.

1.0 ml purified anti guinea pig IgG antibody (0.5 mg/ml) in bor-

ate buffered saline was added to each plate and the plates were

incubated at 4 C for 24 hours. Radioiodinated antibody was added as a tracer to determine the extent of coupling. After incubation, the plates were washed with PBS and stored with PBS containing 15% inactivated FCS and 5.0 mg/ml sodium azide. Free aldehyde groups remaining on the nylon sheet couple to FCS in this step.

2.0 ml of lymph node cell suspension (2.5 x 10^7 cells/ml in PBS + 15% FCS + 0.015 M NaN_3) were added to each IgG plate and the plates were incubated 30 minutes at 4 C with periodic agitation. The plates were then washed three times with 3.0 ml PBS containing 15% FCS and the effluent cells were recovered and washed 3X with PBS + 15% FCS. Viable cell numbers were determined by trypan blue exclusion and aliquots prepared for lymphocyte stimulation, antigen suicide experiments and autoradiography.

XXVII Anti-IgG Autoradiography

Unfractionated (U) cells and fractionated (E or effluent) cells were examined for the presence of surface immunoglobulin using anti-IgG autoradiography by the following procedure: 1 x 10^7 cells in 0.2 ml PBS + 0.015 M sodium azide + 15% FCS were incubated at 4 C for 1 hour in the presence of 7.0 µg ^{125}I labelled sheep anti guinea pig IgG (specific activity 0.36 µCi/µg). The purified sheep anti guinea pig IgG was prepared by Fumio Takei in this laboratory and was shown to be homogenous by immunodiffusion. The cells were washed free of unbound anti IgG by centrifugation through three discontinuous fetal calf serum gradients and slides prepared and developed as previously

described. Immunoglobulin positive cells were identified as those
cells with lymphocytic morphology and having 10 or more grains over-
lying or associated with them. A minimum of 500 cells were counted
for each cell preparation. (Procedure modified from Ada (1).

RESULTS AND DISCUSSION

I Skin Tests.

The ability of OFD, $N_{10}C$, PPD and KLH to elicit immediate or delayed skin reactions was tested in sensitized and unsensitized guinea pigs. The sensitized animals had been immunized 10-14 days earlier with OFD (or $N_{10}C$), and KLH in complete Freund's adjuvant. Antigen preparations were injected in 0.1 ml saline intradermally into the flanks of the animals. An erythema of greater than 6 mm was considered a positive skin reaction as saline controls were always of lesser diameter. Development of a positive reaction after 3 hours indicated the presence of circulating antibody to the antigen (immediate reaction). Skin erythema existing after 24 hours indicated that a cell-mediated reaction to the antigen had occurred (delayed reaction). Animals giving positive delayed reactions were used in subsequent experiments requiring immune animals. Skin test negative guinea pigs were boosted and skin tested 10-14 days later.

In $N_{10}C$ immunized animals, 50 µg $N_{10}C$, $C_{mal}C$ and PPD elicited only delayed reactions indicating the absence of circulating antibody to these antigens (Table II). Several animals failed to respond to $N_{10}C$ and $C_{mal}C$, an observation reflected later in the degree of lymphocyte stimulation found with these guinea pigs. In $C_{mal}C$ immunized animals, delayed responses were found in all cases (Table III).

Animals immunized with both OFD and KLH gave positive delayed

skin reactions with all antigens tested (Table IV). KLH gave both immediate and delayed reactions indicating that both humoral and cellular immunity were induced. Large areas of erythema (up to 50 mm diameter) found in delayed responses to KLH indicated that a high degree of cellular immunity had been induced.

Specificity of the skin reactions was demonstrated by the absence of erythema after skin testing in unimmunized (control) guinea pigs (Table V).

Table II: Skin reactions observed on guinea pigs immunized with $N_{10}C$

| Challenging antigen | Test dose μg per 0.1 ml | Skin Reactions[1] | | Average Diameter of Erythema (mm) (24 hours) |
		Immediate (3 hours)	Delayed (24 hours)	
$N_{10}C$	50	0/14	10/14	10
$C_{mal}C$	50	0/14	9/14	10
PPD	50	0/14	14/14	15
Saline Control	0.1 ml	0/14	0/14	2

[1] Figures represent the ratio of the number of animals showing positive

reactions (> 6mm) to the number of animals tested).

Table III: Skin reactions observed on guinea pigs immunized with $C_{mal}C$

Challenging antigen	Test dose µg per 0.1 ml	Skin Reactions[1]		Average Diameter of Erythema (mm)
		Immediate (3 hours)	Delayed (24 hours)	
$N_{10}C$	50	0/4	4/4	10
$C_{mal}C$	50	0/4	4/4	9
PPD	50	0/4	4/4	18
Saline Control	0.1 ml	0/4	0/4	3

[1] Figures represent the ratio of the number of animals showing positive reactions (> 6mm) to the number of animals tested.

Table IV: Skin reactions observed on guinea pigs immunized with OFD and

KLH

Challenging antigen	μg Test dose per 0.1 ml	Skin Reactions[1]			
		Immediate (3 hours)	Average Diameter of Erythema (mm)	Delayed (24 hours)	Average Diameter of Erythema (mm)
$N_{10}C$	50	0/10		8/10	9
OFD	50	0/18		14/18	10
PPD	50	0/18		16/18	15
KLH	50	10/18	25	16/18	23
Saline Control	0.1 ml	0/18		0/18	3

[1] Figures represent the ratio of the number of animals showing positive

reactions (> 6mm) to the number of animals tested.

Table V : Skin reactions observed on unimmunized guinea pigs

Challenging antigen	Test dose μg per 0.1 ml	Skin Reactions[1]		Average Diameter of Erythema (mm)
		Immediate (3 hours)	Delayed (24 hours)	
$N_{10}C$	50	0/4	0/4	2
$C_{mal}C$	50	0/4	0/4	2
OFD	50	0/2	0/2	2
PPD	50	0/4	0/4	5
KLH	50	0/2	0/2	5
Saline	0.1 ml	0/4	0/4	2

[1] Figures represent the ratio of the number of animals showing positive reactions (> 6mm) to the number of animals tested.

II Lymphocyte Stimulation

Lymphocyte transformation has been assessed by measuring the up-
take of tritiated thymidine into the DNA of lymphocytes undergoing

blastogenesis. The non-specific activation of lymphocytes by mitogens

or the specific activation of sensitized cells by antigens, results in

an increased degree of DNA synthesis prior to cell division. The degree

of DNA synthesis (and thus degree of stimulation) can be quantitated

by comparing the amount of ^{3}H-thymidine incorporated into the DNA of

lymphocyte cultures containing antigen or mitogen to that incorporated

into the DNA of cell cultures without added antigen or mitogen.

Experiments were carried out to determine optimal culture condi-
tions for the stimulation of guinea pig lymph node cells using phyto-

hemagglutinin, concanavalin A, and the protein antigens OFD, $N_{10}C$, PPD

and KLH, employing a microculture system and a semi-automatic multiple

sample harvester.

(a) <u>Stimulation of Guinea Pig Lymph Node Cells by PHA and ConA.</u>

Phytohemagglutinin (PHA) and Concanavalin A (ConA) induce blast-like transformation in T lymphocytes (120) and were thus chosen to investigate optimal parameters for the microculture and stimulation of T lymphocytes.

As the incubation time increased, maximum PHA response was found in cultures containing lower cell density, ie. at day two, maximum PHA response was obtained with 5×10^5 cells/well (Figure 9) at day 3 with 2.5×10^5 cells/well (Figure 10) and at day 4 with 1×10^5 cells/well (Figure 11).

Culture wells with added PHA became quite acid after 24 hours incubation especially with high cell numbers (5×10^5 - 1×10^6/well), suggesting that in these cultures, conditions soon became toxic. This would explain the rapid decrease in lymphocyte response with 5×10^5 cells/well between day 2 and 3. Cultures containing lower cell density reach peak stimulation much later as there are fewer responding cells and the cultures do not become excessively acid until after lengthy incubation. PHA stimulation using 5×10^5 cells/well was chosen as a measure of T cell viability in subsequent experiments.

The response of guinea pig lymph node cells to varying concentrations of ConA is shown in Table VII. A final ConA concentration of 4.0 µg /ml was used in all further tests.
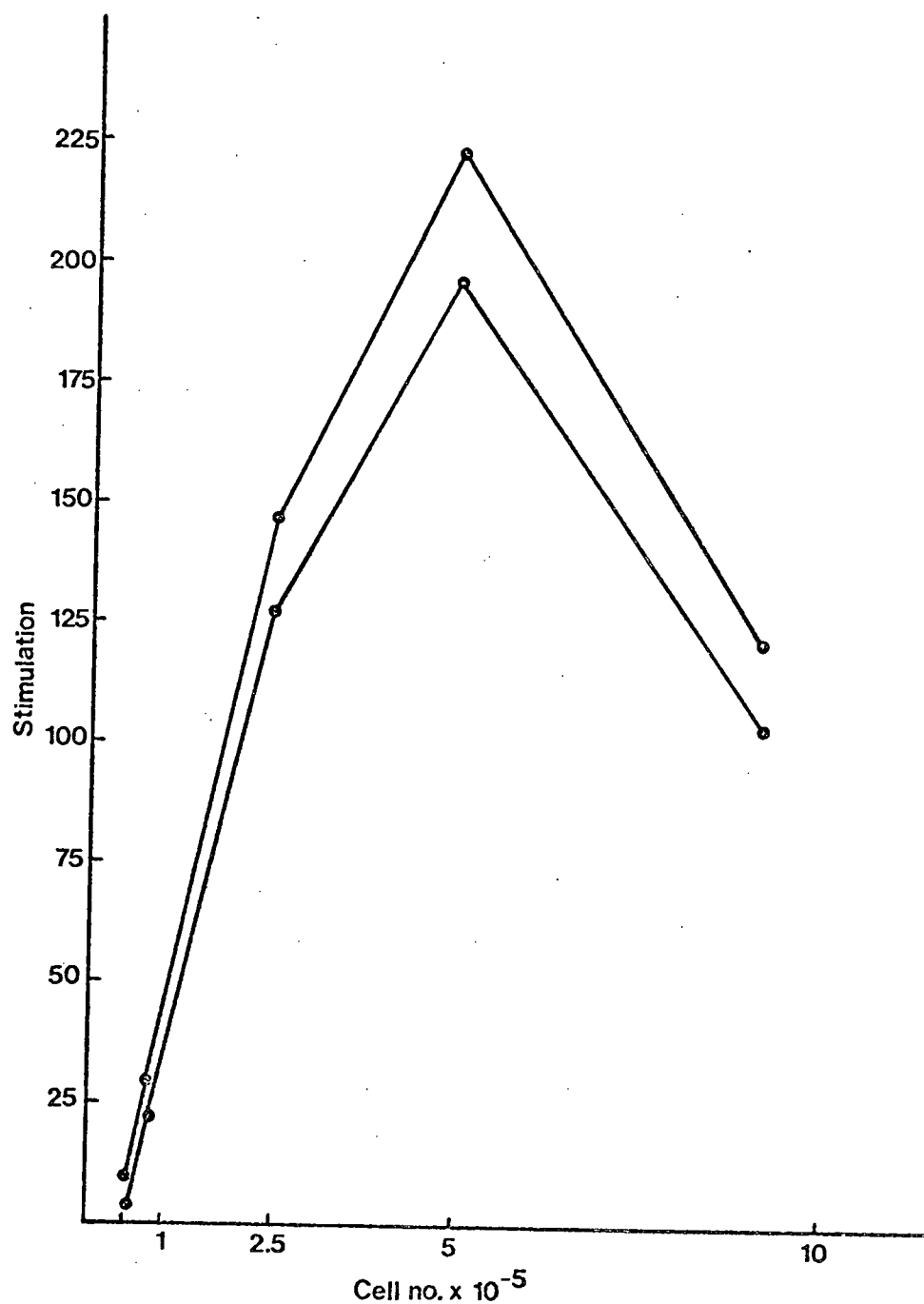
Figure 9 . PHA Stimulation Vs Cell Number.

Stimulation represents the ratio of the average ^{3}H-thymidine incorporation in triplicate cultures with PHA (1/100) present to the average ^{3}H thymidine incorporation in triplicate control cultures without PHA.

Cultures were pulsed with ^{3}H thymidine at 48 hours and harvested 16 hours later. Results from 2 guinea pigs are shown.
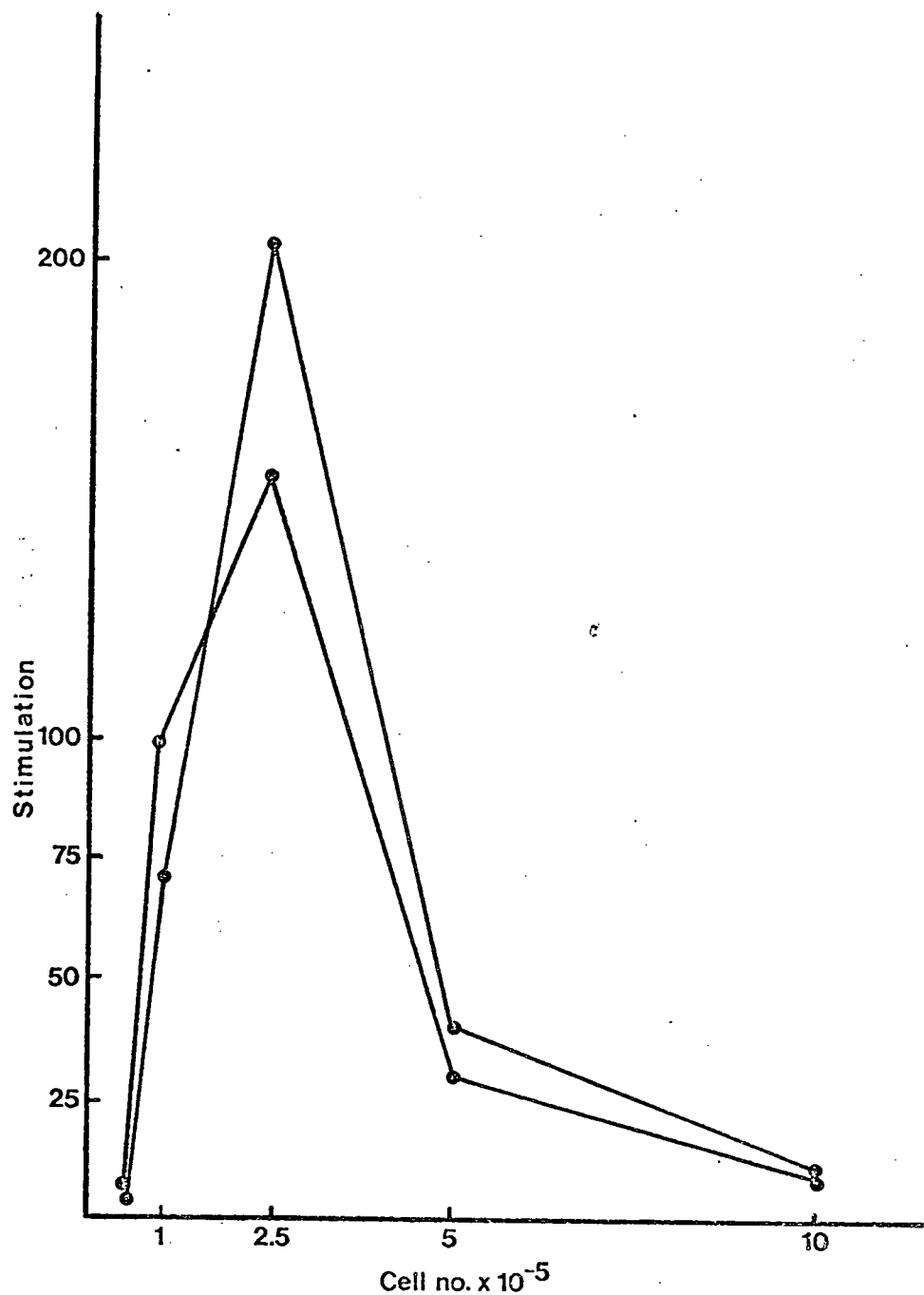
Figure 10. PHA Stimulation Vs Cell Number.

Stimulation: as defined in Figure 9 .

Cultures were pulsed with ^{3}H-thymidine at 72 hours and harvested 16 hours later. Results from 2 guinea pigs are shown.
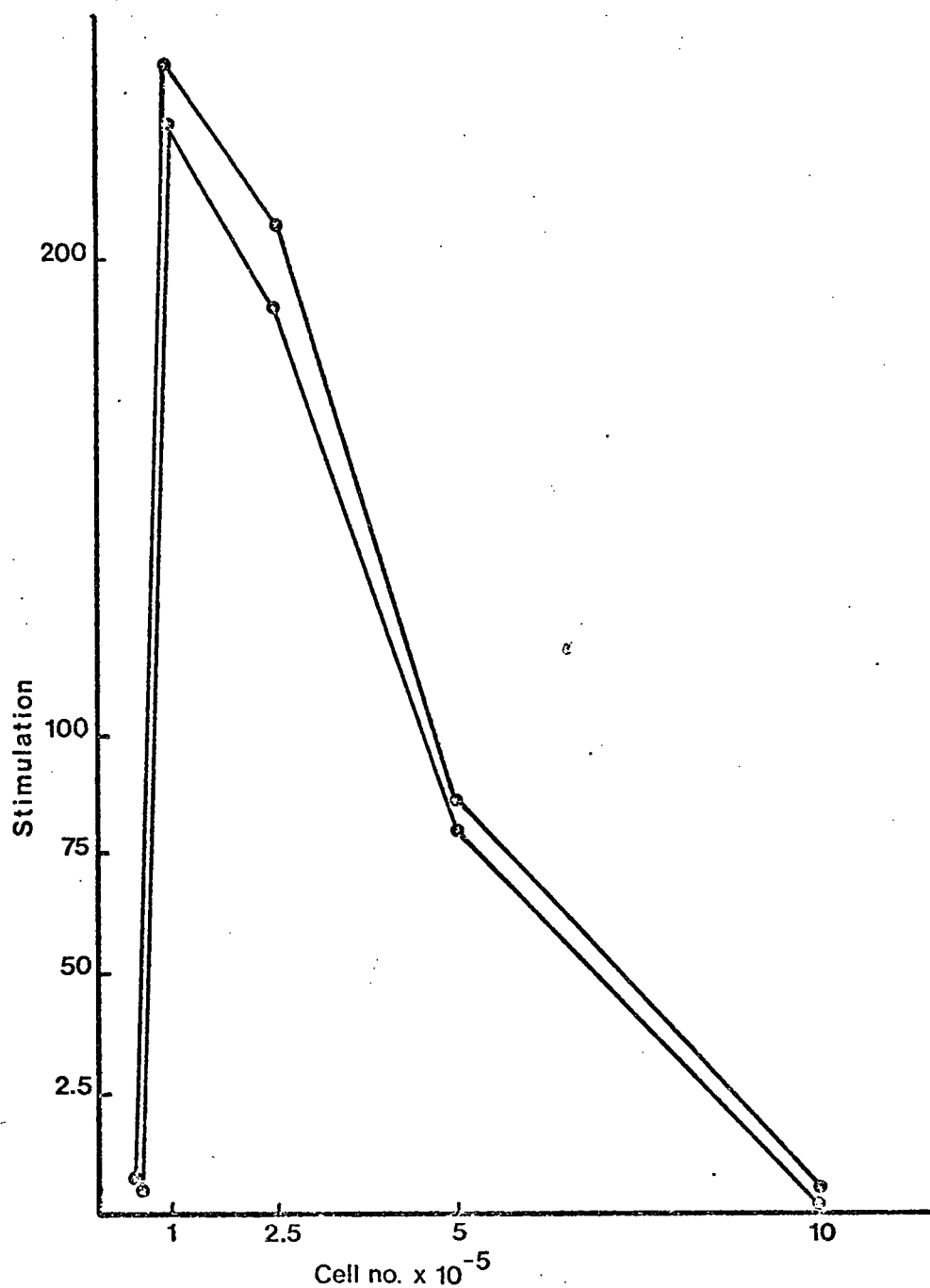
Figure 11.   PHA Stimulation Vs Cell Number.

Stimulation: as defined in Figure 9.

Cultures were pulsed with ^{3}H thymidine at 96 hours and harvested 16 hours later. Results from 2 guinea pigs are shown.

Table VII: ^{3}H-thymidine uptake in guinea pig lymph node cells in response to varying concentrations of ConA

Final ConA Concentration (µg /ml)	CPM (± standard deviation)[1]
0.0	863 (± 176)
1.0	761 (± 86)
2.0	9,061 (± 261)
4.0	25,092 (± 1302)
8.0	24,961 (± 1313)
20.0	8,042 (± 651)
40.0	4,691 (± 402)

[1] Average CPM and standard deviation were calculated from quadruplicate cultures of guinea pig lymph node cells (5 x 10^{5}/well), pulsed with ^{3}H-thymidine after 48 hours incubation and harvested 16 hours later.

(b) Stimulation of Sensitized Guinea Pig Lymph Node Cells by $N_{10}C$

The stimulation of $N_{10}C$ and OFD immunized guinea pig lymph node cells by $N_{10}C$ diluted in RPMI 1640 was tested at final concentrations varying from 12 - 96 µg /ml (Table VIII) A final $N_{10}C$ concentration of 24 µg /ml was used in all subsequent experiments. The effect of varying cell concentrations and incubation period on the $N_{10}C$ response is shown in Figure 12 & 13 respectively. Maximum response was obtained using 5 x 10^5 cells/well and a four-day incubation period.

(c) Stimulation of Sensitized Guinea Pig Lymph Node Cells by OFD.

Stimulation of OFD immune lymph node cells by varying concentrations of oxidized ferredoxin is shown in Table VIII A four day incubation period (96 hours) and a cell concentration of 5 x 10^5 cells/well were used in all tests. The optimum response was obtained with a final OFD concentration of .0008 µ moles (4.8 µg) per ml, and this concentration was used in all subsequent experiments.

(d) Stimulation of Sensitized Guinea Pig Lymph Node Cells by PPD.

All animals were immunized with antigens emulsified in complete Freund's adjuvant (CFA), therefore purified protein derivative (PPD) stimulated cells from immunized animals. (Freund's adjuvant consists in part of M. tuberculosis, the bacterium from which PPD is derived). Optimal stimulation was achieved with a final concentration of 10 µg PPD per ml (Table VIII) As with $N_{10}C$ and OFD, a cell concentration of 5 x 10^5/well yielded maximum stimulation of thymidine uptake (Figure 14).

(e) <u>Stimulation of Sensitized Guinea Pig Lymph Node Cells by KLH</u>.

Of the antigens tested in this study, keyhole limpet hemocyanin

(KLH) induced the greatest thymidine uptake. Varying concentrations

of KLH were tested at day four using 5×10^5 cells/well (Table VIII).

Maximum response was obtained with a final concentration of 20 µg

KLH per ml.

(f) Stimulation of Unimmunized (Control) Guinea Pig Lymphocytes by
     Test Antigens.

The specificity of the antigen preparations used for stimulation

is shown by experiments performed with cells from unimmunized guinea

pigs. PPD was the only antigen causing significant stimulation (Table

IX) which could be attributed to its reported mitogenic properties

for B cells (120). PHA response was used as a positive control in

these experiments.

Table VIII. ^{3}H thymidine incorporation in $N_{10}C$ or OFD/KLH immunized guinea pig lymph node cells in response to varying anti-gen concentrations

Sensitizing Antigen[1]	Challenging Antigen (μg /ml final concentration)		CPM ± standard deviation[2]
$N_{10}C$	$N_{10}C$	96 μg	402 ± 69
		48 μg	481 ± 82
		24 μg	488 ± 64
		12 μg	211 ± 61
		0 μg	206 ± 41
	PPD	40 μg	302 ± 61
		20 μg	841 ± 112
		10 μg	876 ± 102
		2 μg	318 ± 56
		0 μg	326 ± 48
OFD/KLH	OFD	19.2 μg	488 ± 102
		9.6 μg	511 ± 31
		4.8 μg	509 ± 48
		2.4 μg	221 ± 61
		0 μg	246 ± 86
	KLH	100 μg	206 ± 06
		40 μg	408 ± 68
		20 μg	2486 ± 202
		10 μg	1681 ± 142
		0 μg	302 ± 21

[1] Antigens were all injected in CFA resulting in a parallel sensitization to PPD.

[2] Calculated from triplicate cultures of guinea pig lymph node cells (5 x 10^5 cells/well) pulsed with ^{3}H-thymidine at 96 hours and harvested at 112 hours.
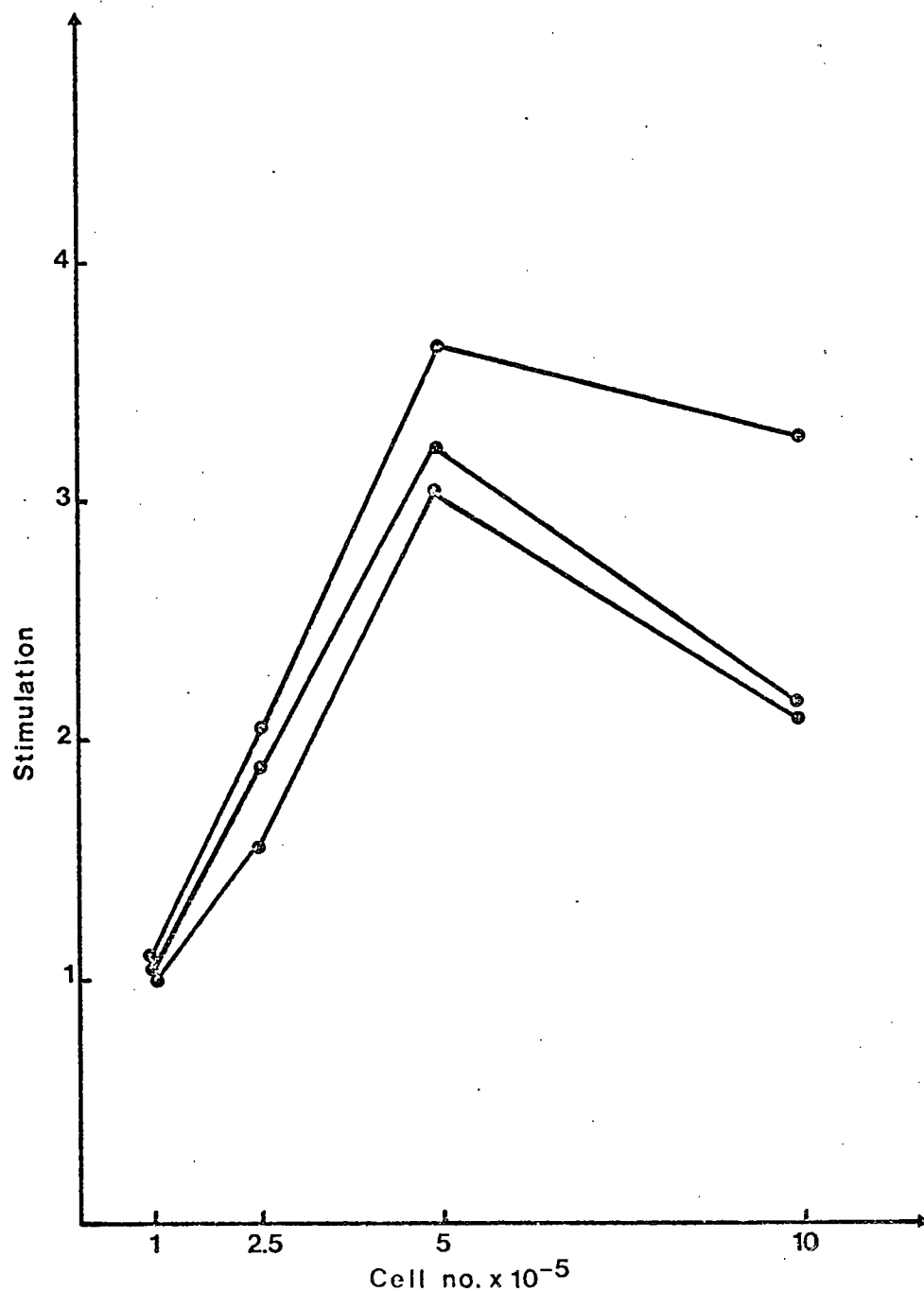
Figure 12. $N_{10}C$ Stimulation Vs Cell Number.

Stimulation: as defined in Figure 9 .

Cultures were pulsed with ^3H thymidine at 96 hours and harvested 16 hours later. Results from 3 guinea pigs are shown.
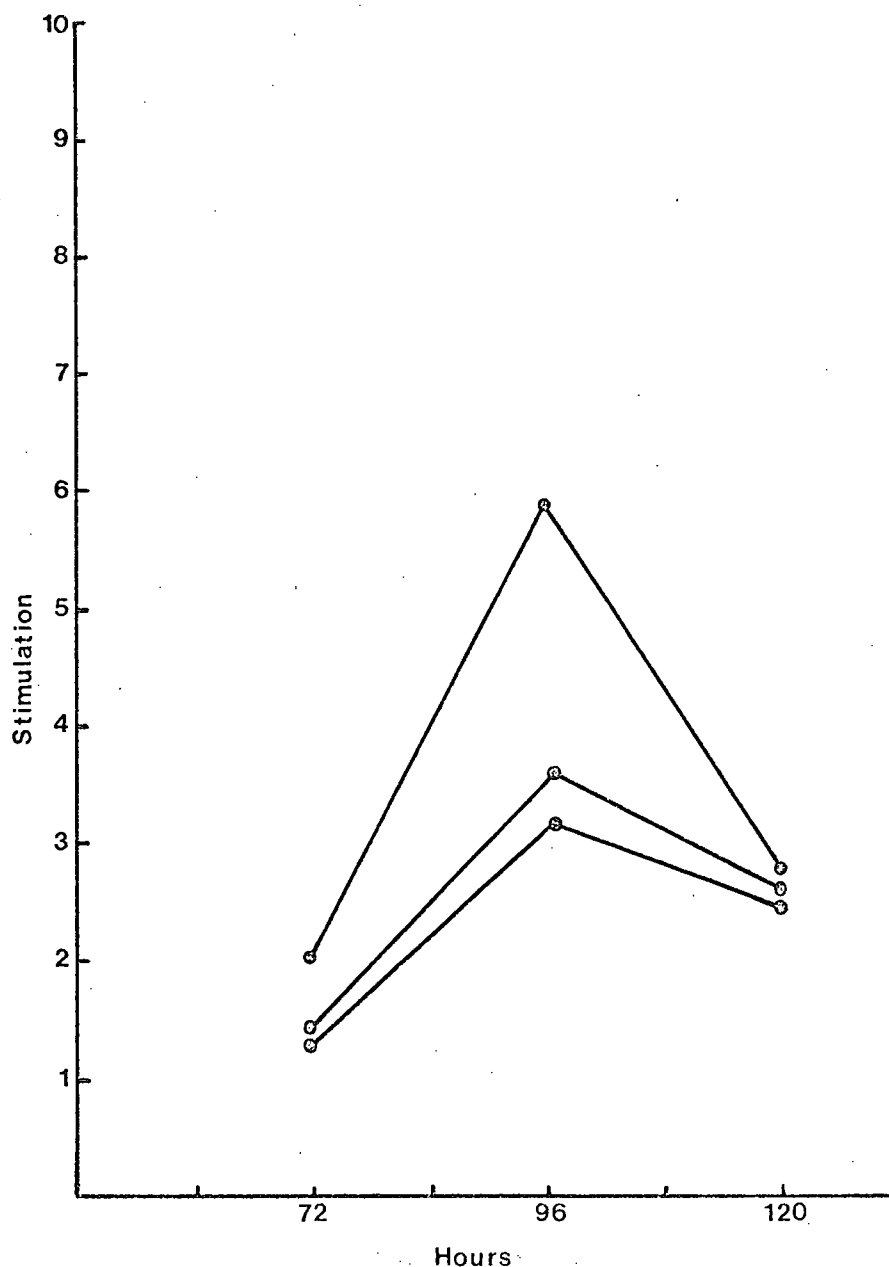
Figure 13. $N_{10}C$ Stimulation Vs Incubation Time.

Stimulation represents the ratio of the average ^{3}H-thymidine incorporation in triplicate cultures containing $N_{10}C$ (24 µg./ml) to the average ^{3}H thymidine incorporation in triplicate control cultures without $N_{10}C$.

Cultures containing 5×10^{5} cells/well were pulsed with ^{3}H thymidine at 3 different time intervals as indicated on the abcissa. Cells were harvested 16 hours later. Results from 3 guinea pigs are shown.
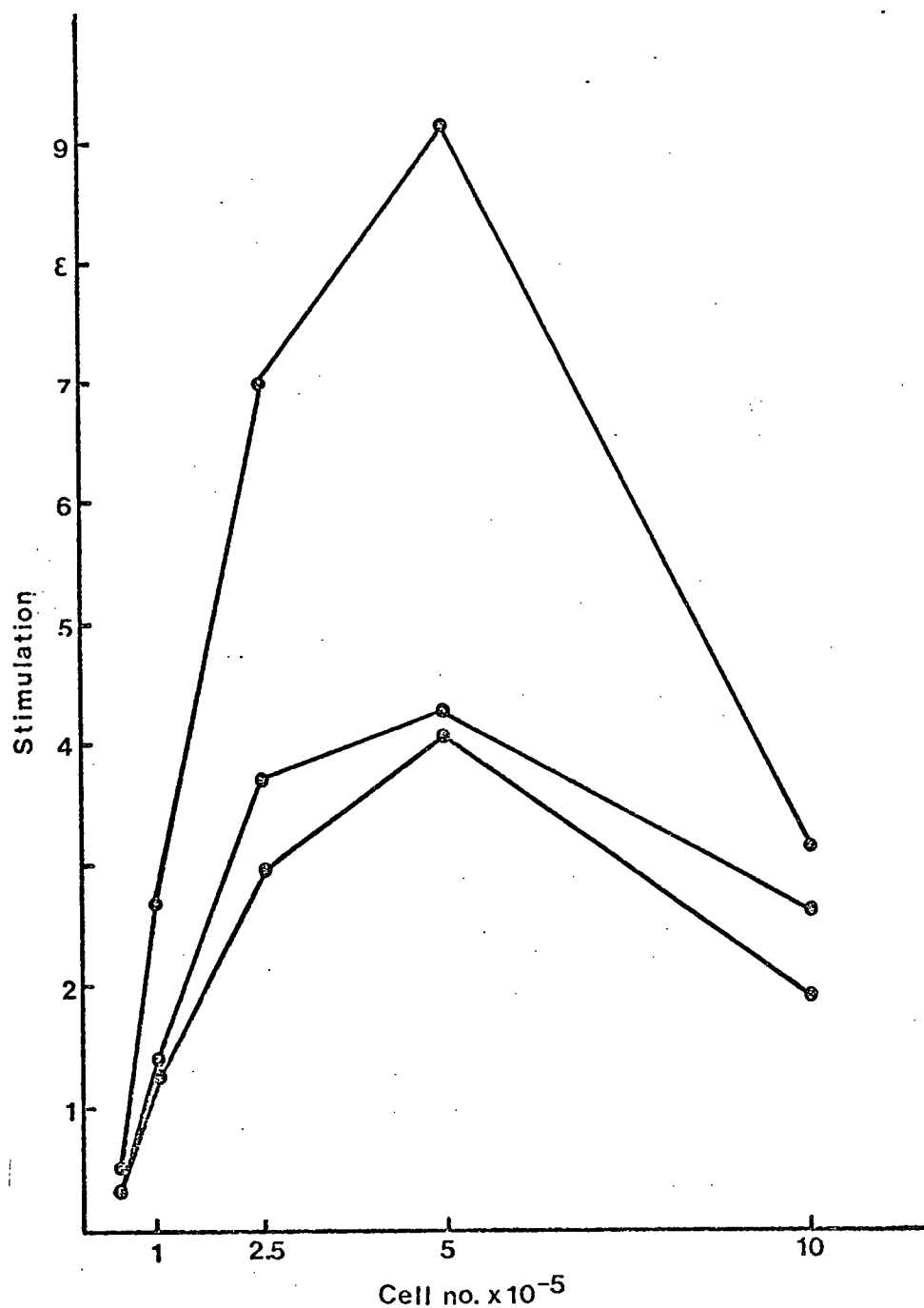
Figure 14. PPD Stimulation Vs Cell Number.

Stimulation represents the ratio of the average ^{3}H-thymidine incorporation in triplicate cultures containing PPD (10 μgm/ml) to the average ^{3}H thymidine incorporation in triplicate control cultures without PPD.

Cultures were pulsed with ^{3}H thymidine at 96 hours and harvested 16 hours later. Results from 3 guinea pigs are shown.

Table IX: ^{3}H-thymidine incorporation in unimmunized (control) guinea
pig lymph node cells in response to antigen preparations
used in lymphocyte stimulation experiments

Challenging Antigen (μg /ml) Final Concentration	Average[1] Stimulation	No. of Animals Tested
$N_{10}C$	1.01±0.06	2
PPD	2.06±0.31	2
OFD	0.98±0.09	2
KLH	0.96±0.10	2
PHA	68.04±8.90	2

[1] Figures were calculated from triplicate cultures of guinea pig lymph
node cells (5 x 10^5/well) pulsed with ^{3}H-thymidine at 96 hours and
harvested at 112 hours. The ratio of ^{3}H-thymidine incorporation in
cultures with antigen present to ^{3}H incorporation in cultures con-
taining no antigen is averaged from experiments with both animals.

Stimulation results compiled from experiments with several animals are given in Table X   Results are presented as the average stimulation ratio plus or minus the standard deviation. KLH consistently induced the greatest thymidine uptake in sensitized animals. PPD also gave good stimulation in all animals tested. The response to $N_{10}C$ was not consistently above 2-fold and depended markedly on the preparation used. For this reason, OFD was used for immunization and stimulation in all later experiments.

(g) Reproducibility

Reproducibility using the microharvester apparatus was tested by labelling, pooling and redistributing PHA and ConA stimulated and unstimulated guinea pig lymph node cells into microtitre wells before harvesting. Results are shown in Table VI. The average percent standard deviation between replicates ranged between 3.5% - 12.5% in unstimulated (control) cultures and between 0.5% - 5.2% in ConA and PHA stimulated cultures respectively. Other reports indicate that an error of 3.5% - 4.5% (standard deviation) is added simply by dispensing MLC samples with Hamilton syringes (49). Maximum error introduced by the labelling and harvesting procedures therefore is in the order of 9.0% average standard deviation.

Reproducibility of the microculture-harvester system is shown in a compilation of data from actual experiments (Table XI).

Table X : Antigen induced ^{3}H-thymidine incorporation in guinea pig

lymph node cell cultures from sensitized guinea pigs

Immunizing Antigen	Stimulating Antigen	Final Antigen Concentration		Average[1] Stimulation	No. of Animals Tested
$N_{10}C$	$N_{10}C$	.01	μmoles/ml	2.31±0.91	10
	PPD	10	μg /ml	3.88±1.00	10
$C_{mal}C$	$N_{10}C$	.01	μmoles/ml	1.48±0.23	5
	PPD	10	μg /ml	3.38±1.06	5
	$C_{mal}C$	.01	μmoles/ml	2.38±0.51	5
OFD, KLH	OFD	.0008	μmoles/ml	2.95±1.13	10
	PPD	10	μg /ml	4.09±1.54	10
	KLH	20	μg /ml	8.55±4.65	10

[1] Figures for average stimulation are derived from the ratio of ^{3}H-thymidine incorporation in triplicate cultures with antigen present to those without for each animal.

Table VI: ^{3}H-thymidine uptake in guinea pig lymph node cell replicate microcultures dispensed with a constant delivery syringe and processed with the microharvester apparatus

Sample Number	Control (CPM)	PHA[1] (CPM)	Sample Number	Control (CPM)	ConA[2] (CPM)
1	1102	29,163	1	762	36,421
2	1412	28,204	2	871	37,402
3	976	31,216	3	804	37,116
4	1111	31,001	4	811	36,902
5	1042	34,696	5	901	35,111
6	1007	28,921	6	799	37,911
7	963	27,041	7	789	37,004
8	915	30,263	8	851	36,991
9	1181	30,211	9	802	37,100
10	1411	29,402	10	807	37,102
11	1002	27,402	11	820	38,009
12	1316	27,826	12	819	37,931
	1119±140	29,612±1554		820±29	37,083±192

Average percent standard Control 12.5
deviation: PHA Stimulated 5.2

Average percent standard Control 3.5
deviation: PHA Stimulated 0.5

[1] PHA (1/100) stimulated and unstimulated (control) guinea pig lymph node cells labelled with ^{3}H-thymidine after 48 hours in culture, dispensed into microtitre plates (5 x 10^5 cells/well) in 0.25 ml volumes using a Hamilton constant delivery syringe.

[2] ConA (4 μg /ml) stimulated and unstimulated (control) guinea pig lymph node cells treated as in 1.

Table XI . Reproducibility of the microculture-harvester system:
^{3}H-thymidine incorporation by microcultures[1] stimulated
with OFD, KLH and PPD.

Experiment[2]	Average CPM (± Standard Deviation)[3]			
	Control	OFD	KLH	PPD
1	402 (2.4)	929 (0.2)	4057 (0.1)	961 (1.0)
2	226 (2.5)	435 (16.5)	442 (29.1)	3298 (11.5)
3	826 (22.0)	21985 (7.5)	17334 (10.6)	44758 (8.6)
4	1900 (10.2)	8361 (12.8)	10548 (9.5)	6378 (13.3)
5	491 (8.6)	1213 (24.0)	1971 (8.2)	1998 (12.1)
Average percent standard deviation	9.1%	12.2%	14.4%	9.3%

[1] Lymph node cells from OFD/KLH immunized guinea pigs (5 x 10^5 mononuclear
cells per well dispensed with a Hamilton constant delivery repeating
syringe) pulsed with ^{3}H-thymidine after 96 hours incubation and harves-
ted 16 hours later with the microharvester apparatus.

[2] Each experiment was performed with cells from a different guinea pig.

[3] CPM ± percent standard deviation calculated from triplicate cultures
from individual guinea pigs.

III Peptide Conjugates

The NH_2-terminal or the COOH-terminal haptenic peptides of ferredoxin were covalently linked to succinylated BSA (s-BSA) producing the conjugates termed N-sBSA and C-sBSA. Because of the probability of multiple binding of such molecules which contain approximately 20 peptides per molecule of s-BSA their avidity should be much greater than that of the peptides alone making their use in autoradiography and antigen suicide experiments preferable. The number of tyrosine residues present in the BSA molecule also enables labelling with ^{125} Iodine to a high specific activity, especially important in achieving sensitivity in autoradiography and effectiveness in antigen suicide experiments. Iodination of the tyrosine in BSA is essential in C-sBSA conjugates as the COOH-terminal peptide contains no tyrosine.

IV Estimation of Coupling

The degree of coupling of peptides was estimated by calculating differences in key amino acids between the conjugates and s-BSA (Table XII). The N-sBSA conjugate was estimated to contain 20 NH_2-terminal peptides per molecule and the C-sBSA conjugate 22 COOH-terminal peptides per molecule.

V Biological Activity Tests

Biological activity of s-BSA and its peptide conjugates was tested in vivo by skin testing and in vitro by production of MIF. The ability of the different preparations to elicit delayed skin reactions in $N_{10}C$

immunized guinea pigs is shown in Table XIII. Inhibition of migration

of sensitized cells induced by the peptide conjugates is shown in

Table XIV. All preparations containing either NH_2-terminal or COOH-

terminal peptides gave positive skin reactions and elicited MIF indi-

cating that the conjugates retained biological activity. The specifi-

city of these reactions is demonstrated by the negative reactions seen

using unconjugated s-BSA alone.

Table XII. Ratios of amino acids[1] in s-BSA and its NH_2-terminal and
COOH-terminal peptide conjugates.

Amino Acid	s-BSA	N-sBSA	C-sBSA
Asp	0.88	2.39[2]	1.04
Thr	0.41	0.49	0.56
Ser	0.33	0.40	0.40
Glu	1.34	1.31	2.30
Pro	0.23	0.19	0.97
Gly	0.35	0.29	0.33
Ala	0.74	2.84	1.32
Val	0.56	0.58	1.13
Met	0.06	0.09	0.03
Ileu	0.20	1.25	0.25
Leu	1.00	1.00	1.00
Tyr	0.32	1.30	0.19
Phe	0.43	0.43	0.53

[1] Calculated using leucine as 1.0 in each analysis.

[2] Figures underlined correspond to the amino acids found in the NH_2-terminal or COOH-terminal peptides.

Table XIII. Biological activity of the peptide conjugates and their
iodinated derivatives - skin reactions observed on
guinea pigs immunized with $N_{10}C$.

Challenging Preparation	Test Dose (μg /0.1 ml)	Delayed Skin Reaction[1] (24 hours)	Diameter of Erythema (mm)
$N_{10}C$	50	2/2	9
NH_2-terminal	50	2/2	10
COOH-terminal	50	2/2	9
s-BSA	50	0/2	3
N-sBSA	50	2/2	12
C-sBSA	50	2/2	10
^{127}I-$N_{10}C$	50	2/2	10
^{127}I-sBSA	50	0/2	5
^{127}I-NsBSA	50	2/2	10
^{127}I-CsBSA	50	2/2	13
Saline (Control)	0.1 ml	0/4	4

[1] Figures represent the ratio of the number of animals showing positive
reactions (> 6 mm) to the number of animals tested.

Table XIV. Migration inhibition[1] by NH_2-terminal and COOH-terminal peptides, sBSA-peptide conjugates, and their iodinated derivatives.[2]

Test Preparation	Test Dose Chamber Concentration		Percent Migration[3]	$\pm$ Standard Deviation
NH_2-terminal	0.05	μmole/ml	67%	$\pm$ 9.1
COOH-terminal	0.05	μmole/ml	72	$\pm$ 4.8
s-BSA	150	μg /ml	106	$\pm$ 19.4
N-sBSA	150	μg /ml	60	$\pm$ 6.9
C-sBSA	150	μg /ml	74	$\pm$ 13.1
^{127}I-Ns-BSA	150	μg /ml	72	$\pm$ 12.1.
^{127}I-CsBSA	150	μg /ml	71	$\pm$ 9.1
Medium (Control)	-		100	-

[1] The migration of spleen cells from $N_{10}C$ immunized guinea pigs measured after 18 hours incubation at 37 C.

[2] ^{127}I-labelled conjugates were tested with the assumption that analogous ^{125}I-labelled proteins would react identically.

[3] Percent migration measured in chambers containing peptides, s-BSA conjugates or their iodinated derivatives compared to control chambers containing culture medium alone. Measurement of migration was performed by determining the rectangular area encompassing the furthest extent of confluent macrophage travel, using a calibrated microscope stage. The average area of two migration patterns from each animal was calculated.

VI Iodination

Iodinated proteins were freed from unreacted Iodine by gel filtration. The elution profile of an $N_{10}C$ Iodination mixture is shown in Figure 15. The labelled $N_{10}C$ was frontally eluted as a single well defined peak while unbound ^{125}I was included in the gel and eluted much later. Between 30% - 50% of the ^{125}I label was associated with the first peak, depending on the nature and quantity of the protein iodinated. Elution profiles of the sBSA conjugates were identical although proportionally more ^{125}I was associated with the protein peak. Experiments using Iodinated molecules as tracers showed that less than 100 ng protein was adsorbed to the Sephadex beads providing the column was washed with FCS prior to sample application. Specific activities (calculated after correcting for column loss) ranged from 100 - 500 μCi per μg for $N_{10}C$ and from 100 μCi- 1 mCi per μg for s-BSA and its peptide conjugates. Iodination of proteins for autoradiography was performed using high concentrations of protein in the reaction mixture (50 - 100 μg /0.1 ml). Specific activities of proteins labelled in this manner ranged from 0.4 to 1.0 μCi per μg.

^{127}I-labelled proteins were collected by pooling tubes corresponding with peak tubes from elution profiles of analogous ^{125}I reaction mixtures. All Iodinated preparations were tested for biological activity with skin tests and MIF tests using ^{125}I-labelled proteins assuming that the analagous ^{125}I-labelled molecules would react identically. Results from these tests indicated that the Iodination procedure did not significantly alter the biological activity of the peptide determinants (Table XIII, XIV).
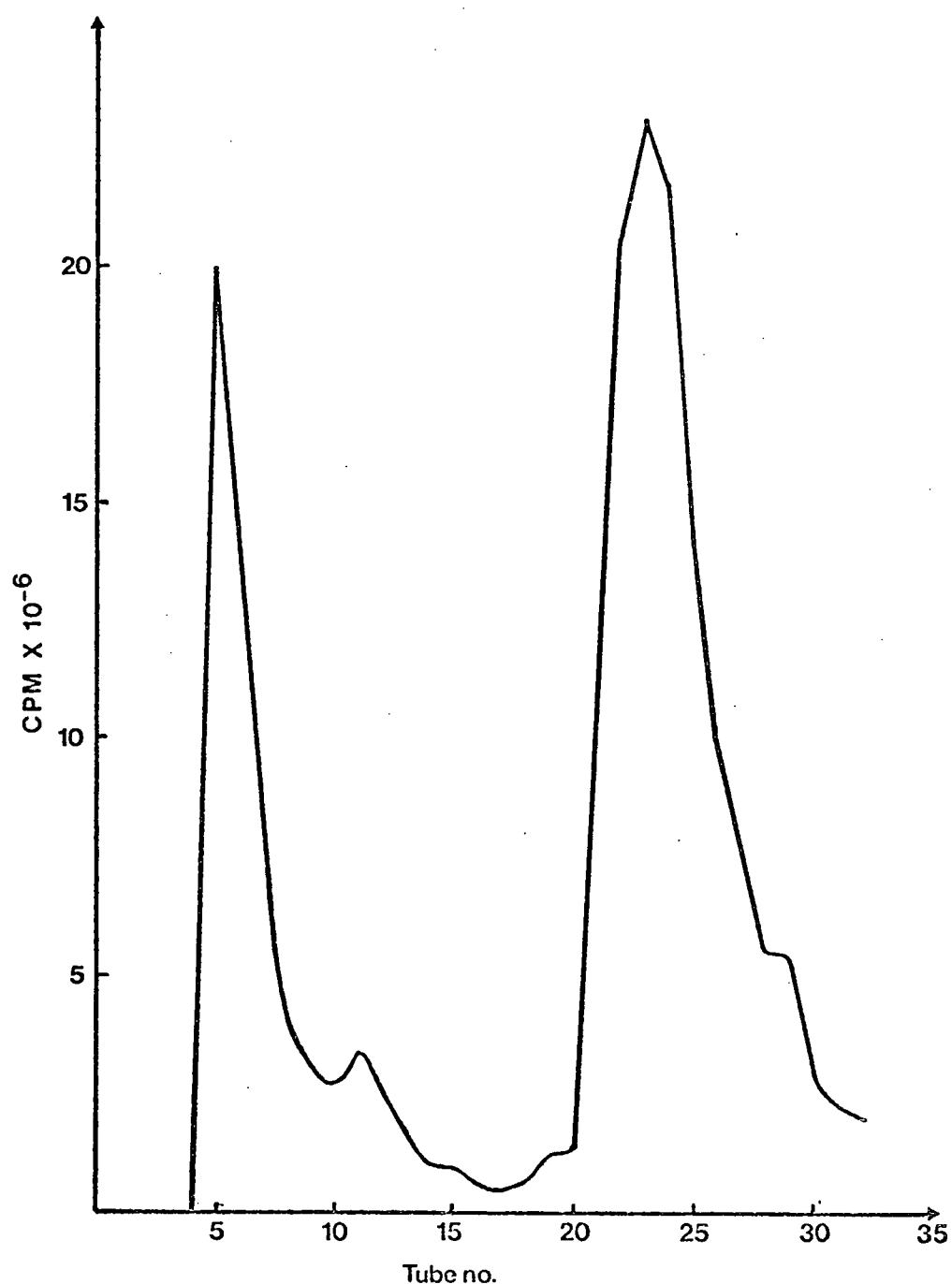
Figure 15. Elution Profile of $N_{10}C$ Iodination Mixture.

Prior to sample application, the column (G-15 Sephadex 1 cm x 10 cm) was equilibrated with 0.01 M phosphate buffer pH 7.5 and 5.0 ml inactivated FCS. Eluting buffer was 0.01 M phosphate pH 7.5 adjusted to 12 drops per minute. Eight drop fractions were collected by hand.

VII Antigen Binding Experiments

The potential of lymphocytes to bind antigen can best be tested in
vitro using a cell suspension in which every cell is exposed to the same
antigen concentration. This approach was first explored by Naor and
Suliztneau (79) who wished to test a tenet of Burnet's Clonal Selection
Hypothesis which proposed that only a small proportion of an animal's
total lymphocyte population would possess receptors for any one antigen
(or antigenic determinant). Autoradiographic examination of cells
exposed in vitro to radioactively labelled antigen revealed only a small
proportion of cells binding antigen. The number of antigen binding cells
observed is influenced by various parameters however and thus asserts a
need for concern about specificity and interpretation (Reviewed by Ada,
1). In the work reported here, antigen binding experiments were
designed to determine the maximum number of cells specifically binding a
given antigen or antigenic determinant while minimizing interference
from non-specific uptake of labelled antigen. The following parameters
were considered in all antigen binding studies:

(a) Uptake of Antigen by Macrophages

Cell suspensions usually contain macrophages and polymorphonuclear
leukocytes which may take up antigens non-specifically. Cohn (20)
reported that phagocytosis by macrophages is inhibited by 15 mM sodium
azide while the binding of antigen to lymphocytes remains unaffected.
Macrophage uptake of $^{125}I\text{-}N_{10}C$ and the iodinated peptide conjugates was

tested both in the presence and absence of sodium azide. The results are shown in Table XV. A high proportion of macrophages from both immune and non-immune animals bound all iodinated proteins tested when incubated in complete medium at 37 C. This uptake was inhibited by incubation at 4 C in the presence of sodium azide (15 mM). Azide was included as a medium component in all subsequent antigen binding experiments.

(b) <u>Uptake of Antigen by Dead Cells</u>.

Damaged cells, cell debris, and isolated nuclei may bind labelled antigen thus making it difficult to determine numbers of antigen binding cells. In order to minimize interference from non-specific uptake of antigen, lymphoid tissues were teased gently apart in the presence of 5% fetal calf serum and the cell suspensions layered onto 100% fetal calf serum. Clumps of aggregated cells and cell debris were allowed to settle out and were discarded. Dead cells and debris were also removed by centrifugation of the cell suspensions through discontinuous fetal calf serum gradients. Cells were counted positive only if they were completely intact with a well defined periphery, evenly stained, possessed a definite lymphoid morphology and had 10 or more grains overlying or associated with them. Emulsion which gave more than 30 background grains per microscope field was discarded.

(c) <u>Effect of Antigen Concentration</u>.

If antigen receptors are generated by a random process (as proposed

in the Clonal Selection Theory) there will be an increasing number of receptors available for binding antigen as the degree of complementarity (between receptors and antigenic determinants) decreases. The frequency distribution of grain counts at a given antigen concentration supports this view as a marked variation in the number of antigen molecules bound per cell is seen. Ada (1) examined the heterogeneity of antigen binding to murine spleen cells using varying concentrations of hemocyanin. Between antigen concentrations of 50 - 1500 ng/ml the number of antigen binding cells increased almost proportionately with the increase in antigen concentration. At a concentration higher than 1500 ng/ml the number of binding cells did not increase.

The effect of increasing concentration of ^{125}I labelled $N_{10}C$ and the BSA conjugates on binding of labelled antigen by $N_{10}C$ immune lymphocytes is shown in Figure 16. Maximum binding was achieved with antigen concentrations of 1.0 μg /0.2 ml (2 x 10^7 cells) and higher for all preparations tested. This concentration (1 μg /0.2 ml) was used in subsequent experiments requiring high levels of radiation (autoradiography and antigen suicide). Since s-BSA and its peptide conjugates were labelled to approximately the same specific activity, the increased counts seen with ^{125}I-N-sBSA and ^{125}IC-sBSA over ^{125}IsBSA incubation mixtures reflects the specificity of the binding. The low counts observed in ^{125}I-N$_{10}$C incubation mixtures may be due to the low specific activity of this antigen. Alternatively, ^{125}I N$_{10}$C which is monovalent for the NHL-terminal and COOH-terminal determinants may be bound less avidly than the polyvalent peptide-sBSA conjugates.

Table XV . Number of antigen binding macrophages in peritoneal

exudates from unsensitized and $N_{10}C$ sensitized guinea

pigs.

Treatment	^{125}I-labelled[1] Protein	Labelled Cells[2] per 10^3	
		Unimmunized	Immunized
Medium + 0.15 M azide 1 hour at 0°C	$N_{10}C$ NsBSA CsBSA	0 1 0	1 1 1
Medium only 1 hour at 37°C	$N_{10}C$ NsBSA CsBSA	502 511 312	471 516 418
Medium only 16 hours at 37°C	$N_{10}C$ NsBSA CsBSA	900 920 817	802 891 916

[1] Specific activities were: $N_{10}C$ 0.8 $\mu Ci/\mu g$, NsBSA 1.4 $\mu Ci/\mu g$ and CsBSA 1.0 $\mu Ci/\mu g$. 1.0 μg ^{125}I-protein was used in each incubation mixture. Slides were exposed for 7 days.

[2] Cells were scored positive if they possessed a definite macrophage morphology and had 10 or more grains overlying or associated with them. 1000 cells were examined in each test.
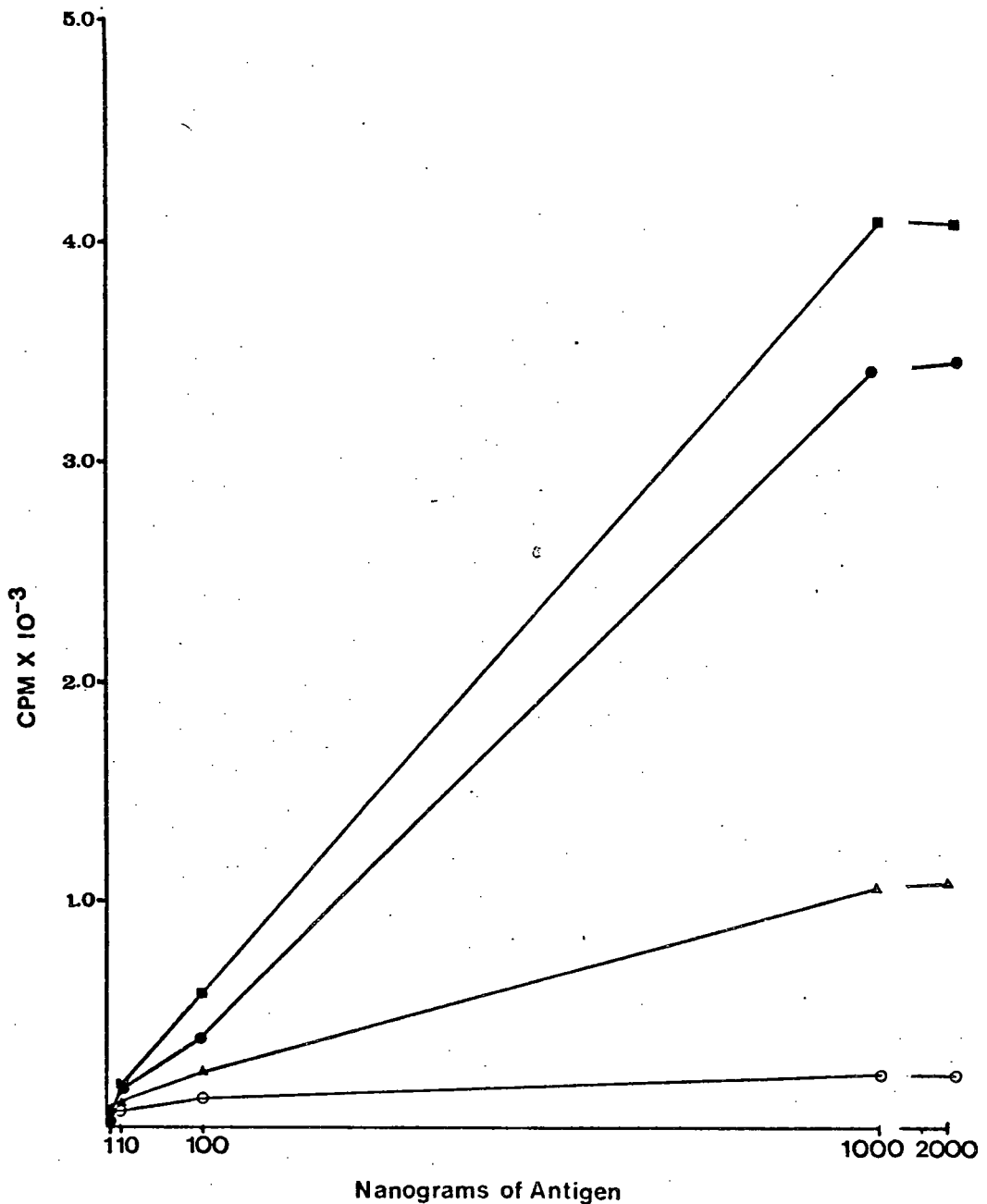
Figure 16. The effect of antigen concentration on the antigen bind-
ing capacity of $N_{10}C$ sensitized guinea pig lymphocytes.

Varying concentrations of ^{125}I labelled proteins were
incubated with 1×10^7 lymphocytes in a total volume of
0.2 ml and the number of bound CPM determined after 1
hour at 4 C.

o——o ^{125}I-sBSA (1.0 μCi/μg)   ▲——▲ ^{125}I-$N_{10}C$ (0.4μCi/μg

●——● ^{125}I-CsBSA(1.0 μCi/μg)   ■——■ ^{125}I-NsBSA(1.0 Ci/μg

(d) Effect of Immunization

The numbers of cells specifically binding $N_{10}C$ and the NH_2-
terminal and COOH-terminal peptide determinants were determined by auto-
radiography using lymph node cells from immunized and unimmunized guinea
pigs. The results are presented in Table XVI. A four to five fold
increase in the number of antigen binding cells was seen in both $N_{10}C$
and OFD immunized animals. The specificity of the binding reaction was
indicated by the absence of cells binding ^{125}I-sBSA. Approximately
equal numbers of lymphocytes were labelled with $N_{10}C$ and the peptide
conjugates, a somewhat disconcerting observation as it was expected
that the number of cells binding $N_{10}C$ would equal the sum of the numbers
binding each determinant. This result parallels the findings of the
batch scintillation counting experiment where cells incubated with ^{125}
$I-N_{10}C$ bound relatively low counts in comparison with those incubated
in the presence of the sBSA conjugates. Low specific activity or low
avidity for cell receptors may explain this observation.

The labelled cells were small to medium sized lymphocytes although
labelled macrophages and dead cells were occasionally seen. A few
lymphocytes with grain counts higher than background were not scored
positive because of the arbitrary limit of a minimum of 10 grains per
positive lymphocyte.

(e) The Effect of B Cell Depletion on Antigen Binding Cells

Unfractionated (U) cells and fractionated (E or eluted) cells from
nylon fibre columns and anti-IgG plates were examined for the presence

of surface immunoglobulin using anti-IgG autoradiography. B cells were equated with immunoglobulin positive cells and were identified as those cells with intact lymphocytic morphology having 10 or more grains overlying or associated with them. The numbers of immunoglobulin positive cells found in unfractionated and fractionated populations are presented in Table XVII. The effect of B cell depletion on numbers of cells binding antigenic determinants of OFD is shown in Table XVIII.

Table XVI. Antigen binding cells in lymph nodes from $N_{10}C$ and OFD

immunized and unimmunized guinea pigs - detection using

low specific activity ^{125}I-labelled molecules.

^{125}I-labelled preparation[1]	Antigen Binding Cells[2] per 10^4 Lymphocytes		
	Unimmunized	$N_{10}C$ Immune	OFD Immune
s-BSA	0	1.0	1.0
N-sBSA	1.0	5.0	6.0
C-sBSA	1.0	5.0	4.0
$N_{10}C$	1.0	4.0	5.0

[1] Specific activities of the iodinated molecules ranged from 0.4 - 1.0

μCi per μg. 1.0 μg was used in each reaction mixture.

[2] 10^4 cells were counted on each autoradiograph slide. Slides were

exposed for 7 days.

Table XVII. The effect of cell fractionation on numbers of immunoglobulin positive guinea pig lymph node cells detected by ^{125}I-anti IgG[2] autoradiography.

Experiment Number	Treatment	Percent Cell Yield	Immunoglobulin Positive[2] Cells/500	Percent Positive Cells
1	none	100	204	40.8
	nylon fibre	22.0	13	2.6
2	none	100	224	44.8
	nylon fibre	20.2	12	2.4
3	none	100	220	44.0
	nylon fibre	16.0	8	1.6
4	none	100	229	45.8
	anti IgG plate	59.0	42	8.4
5	none	100	211	42.2
	anti IgG plate	54.0	71	14.2

[1] Specific activity of the ^{125}I-labelled sheep anti-guinea pig IgG was 0.36 μCi/μg. 7.0 μg was incubated with 10^7 cells in each experiment.

[2] Cells were identified as immunoglobulin positive if they possessed intact lymphoid morphology and had 10 or more grains overlying them or immediately adjacent. 500 cells were counted on each slide.

TableXVIII. The effect of B cell depletion[1] on the number of antigen binding cells in OFD sensitized guinea pig lymph node cells - detection using high specific activity ^{125}I-labelled molecules.

Experiment Number	Cells	Percent Ig Bearing Cells[2]	^{125}I-labelled Preparation[3]	ABC[4]/5 x 10^3 Lymphocytes			
			Grain Number -	(10-20)	(21-30)	(> 30)	Total
1	Unseparated	40.8	sBSA	3	2	0	5
			NsBSA	12	4	0	16
			CsBSA	15	3	0	18
	Eluted	2.6	sBSA	2	1	0	3
			NsBSA	14	1	0	15
			CsBSA	16	1	0	17
2	Unseparated	44.8	s-BSA	0	1	1	2
			N-sBSA	18	4	0	22
			C-sBSA	14	2	2	18
	Eluted	2.4	s-BSA	1	1	0	2
			N-sBSA	14	5	0	19
			C-sBSA	14	2	0	16

[1] B cells were depleted using nylon fibre columns.

[2] Detected by anti-IgG autoradiography.

[3] Specific activities ranged between 196 - 300 μCi/μg.

[4] 5 x 10^3 cells were counted on each autoradiograph slide. Slides were exposed for 7 days.

VIII Immunological Significance of Antigen Binding Cells

(a) Detection of Cell Death by Autoradiography

The functional significance of antigen binding cells was investigated using the radioactive antigen suicide technique in an attempt to inactivate lymphocytes binding the antigen $N_{10}C$ or either the NH_2-terminal or COOH-terminal antigenic determinants.

The antigen suicide technique depends on the marked sensitivity of lymphocytes to ionizing radiations (55). If the radiation from bound antigen leads to death of the cell, the outcome might be detectable by autoradiography. The number of cells killed by radiation from bound ^{125}I-NH_2-terminal and ^{125}I-COOH-terminal antigenic determinants was estimated by comparing autoradiograph slides before and after antigen suicide (Table XIX). A marked decrease in the number of antigen binding cells was seen after antigen suicide. The greatest decrease was seen in cells with higher grain numbers, a result compatible with the idea that cells binding most antigen would be most easily killed. Cells inactivated but not killed would still be observed as antigen binding cells, likewise with cells not lysed by the radiation. Shedding of hot antigen may account for these observations although it is unlikely under the conditions used.

(b) Lymphocyte Stimulation - Low Dose Antigen Suicide

The functional activity of cell populations (before and after antigen suicide) was tested in vitro using antigen induced lymphocyte stimulation as an index of cellular immunity. Initial experiments used low doses of ^{125}I-labelled $N_{10}C$ (100 ng/5 x 10^7 cells) with the object of

Table XIX. Antigen binding lymph node cells from OFD immunized guinea pigs - detection using high specific activity ^{125}I-labelled molecules before and after antigen suicide.

Experiment Number	^{125}I-labelled Preparation[1]	Treatment of Cells[2]	Antigen Binding Cells[3]/5 x 10^3 Lymphocytes			
			Grain Number - (10-20)	(21-30)	(> 30)	Total
1	sBSA	Slides	0	1	1	2
	N-sBSA	made at	18	4	0	22
	C-sBSA	zero time	14	2	2	18
	sBSA	Stored	2	0	1	3
	N-sBSA	24 hours	8	0	0	8
	C-sBSA	at 4 C	3	0	0	3
2	sBSA	Slides	3	2	0	5
	N-sBSA	made at	12	4	0	16
	C-sBSA	zero time	15	3	0	18
	sBSA	Stored	0	0	0	0
	N-sBSA	24 hours	4	1	0	5
	C-sBSA	at 4 C	7	0	0	7

[1] Specific activities of iodinated molecules ranged from 196-300 μ Ci/μg.

[2] Cells were spread onto slides immediately after labelling or after allowing radiation death by incubating for 24 hours at 4 C.

[3] 5 x 10^3 cells were counted on each autoradiograph slide. Slides were exposed for 7 days before developing.

eliminating only highly avid antigen binding cells and minimizing non-specific cell inactivation. The results are shown in Table XX. After suicide, the average proliferative response to $N_{10}C$ was 32.7% of the untreated control response. The decreased $N_{10}C$ response was highly significant as shown by T test ($p < 0.01$). Inactivation was specific since the decrease in the PPD response was not statistically significant ($p > 0.10$).

If the in vitro proliferative response to OFD involves the co-operation of cells directed to each of the antigenic determinants, then the inactivation of cells binding either determinant should eliminate the response. To test this, specific inactivation of cells binding the COOH-terminal determinant was attempted using low doses of ^{125}I-CsBSA (Tables XXI and XXII). Thymidine uptake induced by $N_{10}C$ and $C_{mal}C$ was decreased significantly ($p < 0.005$) in both $C_{mal}C$ and $N_{10}C$ sensitized cells indicating that COOH-terminal binding cells function in the stimulation response to these antigens and are inactivated by the iodinated COOH-terminal s-BSA conjugate. The lack of significant decrease in response to PPD ($p > 0.10$) demonstrates the specificity of killing with ^{125}I-CsBSA.

High doses (1.0 µg /2 x 10^7 lymphocytes) of ^{125}I-labelled antigens were used in an attempt to further decrease the stimulation response in all subsequent experiments. ^{125}I-sBSA treated cells were included as an additional control, all stimulation ratios were based on these cells treated with non-specific radiation.

Specific reduction of the OFD response (Table XXIII) was found

using both ^{125}I-NsBSA and ^{125}I-CsBSA (p << 0.005 and P << 0.01 respectively) while responses to the control antigen KLH (Table XXIV) (P = 0.10 and 0.025 respectively) were not significantly reduced.

Cell mixing experiments were also performed in order to test cell-cell interaction, ie. mixing of a cell population suicided with the NH_2-terminal determinant with a population suicided with the COOH-terminal determinant should restore the response to control levels if cell synergy occurs. If no synergy occurs, the stimulation value obtained by mixing the two populations is expected to be the average of the sum of the contribution of two populations. This expected stimulation ratio was compared with the observed values in all cell mixing experiments. Chi-square analysis of expected and observed stimulation ratios in mixed populations showed that the OFD response was significantly higher (p << 0.005) than would be expected in the absence of synergy (Table XXIII). The failure to restore the OFD response fully may be due to decreased numbers of active NH_2-terminal and COOH-terminal binding lymphocytes in the suicided cell preparations brought about by non-specific irradiation from the bound antigen. The observed KLH responses were significantly lower than would be expected (p << 0.005), (Table XXIV). This indicates inhibition of response rather than enhancement as seen with OFD stimulation in mixed populations. The reason for this inhibition is unclear although the reduced KLH response may reflect poor culture conditions, perhaps due to toxic products contributed by each suicided population.

(c) Lymphocyte Stimulation Using B Cell Depleted Populations - High
Dose Antigen Suicide

The effect of antigen suicide on lymphocyte stimulation in B cell

depleted populations is shown in Table XXV (OFD stimulation) and Table

XXVI (KLH stimulation). Specific depression of the OFD response was

achieved with ^{125}I-NsBSA and ^{125}I-CsBSA (p << 0.005 in both cases)

while the response to KLH was not significantly reduced (p > 0.10 in

both cases). Cell mixing experiments again indicated synergy between

the two populations in the response to OFD (Table XXV). The differ-

ences in observed and expected values for KLH stimulation were again

statistically significant but were lower in 3 out of 5 experiments

(Table XXVI).

Incubation of cell populations in serum-free medium after antigen

suicide resulted in a decrease in ^{125}I counts bound to the cells.

Counts remaining after this incubation usually were in the order of

10% of the total counts before incubation in the serum-free medium

(approximately 100-200 cpm/5 x 10^5 cells).

Table XX . Antigen stimulation of ^{3}H-thymidine incorporation in cultures of $N_{10}C$ sensitized guinea pig lymph node cells treated with low doses[1] of high specific activity $^{125}I-N_{10}C$.

Experiment Number	Stimulating Antigen – Treatment of Cells –	$N_{10}C$		PPD	
		Untreated[2]	$^{125}I-N_{10}C$[3] %	Untreated	$^{125}I-N_{10}C$ %
1		1.28	0.0	1.31	96.7
2		2.07	55.1	1.60	96.6
3		2.11	43.2	2.10	71.82
	Average:		32.7 ± 28.9%		88.4 ± 14
	P Values:	P < 0.01		P > 0.10	

[1] Antigen dose used was 100 ng/5 x 10^5 lymphocytes. The specific activity of the $^{125}I-N_{10}C$ preparations ranged from 100-400 μCi/μg

[2] Ratio of ^{3}H-thymidine incorporation in triplicate cultures containing antigen to those without antigen. PPD (10 μg /ml) and $N_{10}C$ (24 μg /ml) were used for stimulation.

[3] Average percent of control antigen response over background after treating cells with high specific activity $^{125}I-N_{10}C$. ie. $\frac{\text{stimulation ratio suicided cells} \quad -1.0}{\text{stimulation ratio untreated cells} \quad -1.0}$ X 100%.

Table XXI. Antigen stimulation of ^{3}H-thymidine incorporation in cultures of $N_{10}C$ sensitized lymph node cells treated with low doses[1] of high specific activity ^{125}I-CsBSA.

Experiment Number	Antigen (Stimulation) - Treatment of Cells -	$N_{10}C$		$C_{mal}C$		PPD	
		Untreated[2]	^{125}I-CsBSA[3] %	Untreated	^{125}I-CsBSA %	Untreated	^{125}I-CsBSA %
1		3.36	47.0	1.36	0.0	-	-
2		1.28	0.0	-		1.31	225
3		2.07	40.1	-		1.60	96.6
4		4.95	54.6	-		7.86	112.7
5		2.10	14.5	1.32	0.0	2.73	124.8
	Average:		31.2 ± 22.7%		0.0%		139.7 ± 57.9%
	P Values:		P << 0.005				P > 0.10

[1] Antigen dose used for suicides was 100 ng/5 x 10^5 lymphocytes. Specific activity of the ^{125}I-CsBSA preparations ranged from 100 - 500 μc/μg.

[2] Ratio of ^{3}H-thymidine incorporation in triplicate cultures containing antigen to those without antigen. Antigen concentrations used were: $N_{10}C$ 24 μg /ml, $C_{mal}C$ 24 μg /ml and PPD 10 μg /ml.

[3] Average percent of control antigen response over background after treating cells with high specific activity ^{125}I-CsBSA as defined in Table XX .

Table XXII. Antigen stimulation of ^{3}H-thymidine incorporation in cultures of $C_{mal}C$ sensitized guinea pig lymph node cells treated with low doses[1] of high specific activity ^{125}I-CsBSA.

Experiment Number	Stimulating Antigen –	$C_{mal}C$		PPD	
	Treatment of Cells –	Untreated	^{125}I-CsBSA %	Untreated	^{125}I-CsBSA %
1		2.25	30.4	1.54	68.5
2		3.24	40.2	1.63	68.2
3		1.92	48.9	3.56	26.8
	Average:		39.8 ± 9.3%		54.5 ± 23.9
	P Values:		P << 0.005		P >> 0.025

1, 2, 3, as in Table XXI.

Table XXIII. OFD stimulation of ^{3}H-thymidine incorporation in cultures of OFD sensitized guinea pig lymph node cells treated with high doses[1] of high specific activity ^{125}I-sBSA and peptide conjugates.

Experiment Number	Treatment of Cells:-	None[2]	^{125}I-sBSA[3]	^{125}I-NsBSA[4]	^{125}I-CsBSA[5]	Mixed[6] N and C	
						Expected	Observed
1		1.87	100 %	75.8%	108 %	91.9%	68.9 %
2		2.09	100	0.0	25.7	25.7	39.51
3		2.31	100	15.3	69.5	42.4	5.39
4		1.93	45.1	83.3	135.7	109.5	190.4
5		1.61	111.4	60.3	11.8	35.9	69.1
6		1.89	89.9	63.8	65.0	64.4	70.0
7		5.86	89.9	90.6	90.4	90.5	167.2
8		4.40	111.5	58.8	74.7	66.7	77.5
9		2.02	80.4	42.7	31.7	37.2	46.3
10		3.71	95.9	56.1	65.8	61.0	71.5
	Average:		92.4 ± 19.1	54.6 ± 28.6	67.8 ± 38.0	62.5 ± 27.8	80.6 ± 56.3
	P Values:			P << 0.005	P << 0.01		P << 0.005 (Chi square analysis)

[1] Antigen dose used was 1.0 µg /2 x 10^7 lymphocytes. Specific activities of labelled preparations ranged from 200 - 1000 µCi/µ**g**

[2] Ratio of ^{3}H-thymidine incorporation in triplicate cultures containing OFD (4.8 µg /ml) to those without OFD.

[3] Average percent of control OFD response over background after treating cells with high specific activity ^{125}I-sBSA.

[4] Average percent of ^{125}I-sBSA control response over background after treating cells with high specific activity ^{125}I-NsBSA.

[5] As in 4 except cells were treated with high specific activity ^{125}I-CsBSA.

[6] Percentage of the ^{125}I-sBSA control OFD response of a mixture of ^{125}I-NsBSA and ^{125}I-CsBSA treated cells. The expected value is calculated as the average of the sum of the contribution of the two populations if no synergy occurs.

Table XXIV. KLH stimulation of ^{3}H-thymidine incorporation in cultures of KLH sensitized guinea pig lymph node cells treated with high doses[1] of high specific activity ^{125}I-sBSA and peptide conjugates.

Experiment Number	Treatment of Cells:-	None[2]	^{125}I-sBSA[3]	^{125}I-NsBSA[4]	^{125}I-CsBSA[5]	Mixed[6] Expected	N and C Observed
1		9.63	8.24- 83.8%	92.4%	128.7%	110.5%	95.9%
2		1.92	1.92-100.0	176.0	147.8	161.9	147.8
3		10.61	10.61-100.0	49.0	83.4	66.1	39.5
4		14.60	10.67- 70.7	63.2	81.0	72.1	63.5
5		10.02	9.02- 88.9	170.8	102.1	136.4	103.6
6		9.06	9.10-100.5	99.0	115.5	107.2	121.1
7		11.93	11.90- 99.7	89.9	88.0	88.9	62.7
8		3.35	3.12- 90.21	91.0	92.5	91.7	96.2
9		2.59	1.58- 36.4	112.0	124.1	118.0	189.6
10		3.77	3.11- 76.1	95.3	95.7	95.4	94.3
	Average:		84.6%	103.8%	105.9%	104.8%	101.4%
	P Values:			$P = 0.10$	$P = 0.025$		$P < 0.005$ (Chi square analysis)

[1] As in Table XXIII.

[2-6] As in Table   except the stimulating antigen was KLH (20 µg /Ml).

Table XXV. OFD stimulation of ^{3}H-thymidine incorporation in B cell depleted[7] cultures of OFD sensitized guinea pig lymph node cells treated with high doses[1] of high specific activity ^{125}I-sBSA and peptide conjugates.

Experiment Number	Percent[8] B Cells	Treatment of Cells:-	None[2]	^{125}I-sBSA[3]	^{125}I-NsBSA[4]	^{125}I-CsBSA[5]	Mixed[6] Expected	N and C Observed
1	2.6		1.80	108.7	50.6%	19.5 %	35.0 %	28.7 %
2	2.4		2.07	100.0	0.0	(119.6)**	(119.6)*	(165.4)*
3	1.6		3.36	95.8	50.4	50.0	50.2	62.4
4	8.4		2.26	86.5	48.6	29.4	38.9	88.0
5	14.2		3.11	95.3	34.3	39.3	36.8	51.2
		Average:		97.3 ± 8.0	36.6 ± 21.6	34.6 ± 13.0	40.2 ± 6.9	57.5±24.6
		P Values:		P << 0.005	P << 0.005		P << 0.005 (Chi square analysis)	

1-6 As in Table XXIII.

7 Cells in experiment 1-3 were depleted of B cells by passage through nylon fibre columns, and in experiments 4-5 by adherence to anti-IgG plates.

8 As detected by ^{125}I anti-IgG autoradiography.

* Not included in Chi square analysis.

** Not included in average.

Table XXVI. KLH stimulation of ^{3}H-thymidine incorporation in B cell depleted[7] cultures of KLH sensitized guinea pig lymph node cells treated with high doses[1] of high specific activity ^{125}I-sBSA and peptide conjugates.

Experiment Number	Percent[8] B Cells	Treatment of Cells:-	None[2]	^{125}I-sBSA[3]	^{125}I-NsBSA[4]	^{125}I-CsBSA[5]	Mixed[6] Expected	N and C Observed
1	2.6%		2.68	95.8%	100.0 %	99.3%	99.7 %	93.2 %
2	2.4		3.39	67.3	104.9	117.3	(111.1)*	(18.0)*
3	1.6		7.02	109.8	66.7	66.7	66.7	75.0
4	8.4		8.49	71.0	57.33	56.3	56.8	70.3
5	14.2		7.11	98.5	101.5	98.1	99.8	98.3
		Average:		88.5 ± 18.4	86.0 ± 22.3	87.5 ± 25.2	86.8 ± 23.6	84.2 ± 13.6
		P Values:		$p \gg 0.10$	$p \gg 0.10$			$p < 0.05$

[1-6] As in Table XXII except KLH (20 µg /ml) was the stimulating antigen.

[7-8] As in Table XXV.

* Not included in Chi square analysis.

IX Concluding Discussion

The binding of antigen is believed to be the initial step in the complex sequence of events leading to an immune response specific for the inducing antigen and has led many investigators to examine and define the different parameters affecting the antigen binding reaction. A major shortcoming of most of these investigations is the failure to consider the concept that the antigens used possess several, if not many, different antigenic determinants. Presumably these antigens can react with cells possessing specific receptors for any one of the antigenic determinants. The number of cells specifically binding a particular antigenic determinant would therefore be much less than the number binding an antigen containing multiple determinants, an idea which helps explain the observed high frequency of antigen binding cells in virgin populations. While attempts to determine the frequency of antigen binding cells for a single antigen have been successful, a reliable estimate of functional binding cells is hampered by cross-reactivity and binding of determinants on the antigen which do not invoke an immune response. Studies on the diversity of receptors on cell surfaces are also made difficult by employing antigen binding experiments using multideterminant antigens.

The aim of this thesis was to examine cells binding the antigenic determinants of C. pasteurianum oxidized ferredoxin and to study their functional contribution in the cell-mediated immune response to the ferredoxin molecule and its synthetic analog $N_{10}C$. It was hoped that binding studies using highly defined antigenic determinants would allow

greater definition of the significance of antigen binding cells than has been previously available using undefined antigens.

Antigen Binding Macrophages

Much recent work has shown that macrophage bound antigen elicits cell-mediated immunity(102,114,129). It is clear that macrophages do not determine the specificity of the immune response and there is evidence that the extent to which these cells are needed depends upon the nature of the antigen employed (131). Recent work by Rosenthal and Shevach (102, 114)demonstrated that the recognition of soluble protein antigens by guinea pig T lymphocytes requires the presentation of antigen on histocompatible macrophages, and that this interaction between macrophage and T lymphocyte can be blocked by alloantisera, implicating the existence of a specific associative event between macrophage bound antigen and T lymphocytes.

The _in vitro_ results in this thesis showed that macrophages from immune and non-immune guinea pigs bound radiolabelled NH_2-terminal and COOH-terminal antigenic determinants from the oxidized ferredoxin molecule. The binding was energy dependent and non-specific, as up to 92% of macrophages became labelled with the iodinated peptide conjugates.

Further work involving macrophage removal is necessary to determine the functional involvement of macrophages in the cellular response to OFD.

Antigen Binding Lymphocytes

Lymphocytes binding the peptide determinants were found in both

immunized and unimmunized guinea pigs. Since all binding experiments were performed under conditions inhibiting pinocytosis, it seems likely that the binding of determinants was due to the energy-independent association of the determinants with specific receptors on the cell surface. The frequency of cells binding a given determinant was 10^{-4} in unimmunized animals and increased 4-6 fold in OFD and $N_{10}C$ immunized guinea pigs. This frequency is rather higher than expected although it must be considered that the system used relatively high concentrations of labelled conjugates and that high avidity binding would be expected with multideterminant molecules. No significant binding differences between the NH_2-terminal and COOH-terminal determinants were found. The absence of cells binding radiolabelled s-BSA supports the view that specific binding was being observed.

The use of high specific activity radiolabelled molecules in antigen binding studies revealed an important observation. The frequency of determinant binding lymphocytes increased several fold when high specific activity molecules (100 - 300 $\mu Ci/\mu g$) were used, thereby detecting antigen binding cells that would have gone undetected using standard techniques.

Antigen Binding T and B Lymphocytes

Specific T and B lymphocytes can be inactivated by highly radioactive antigen indicating that both T and B cells have antigen specific receptors (9,100). Thus labelled cells observed by autoradiography after incubation of lymphocytes with radioactive antigen of high specific activity should represent a mixture of specific T and B cells. These T and B

subpopulations of lymphocytes can be distinguished by anti-immunoglobulin antisera; only B cells bind sufficient antibody to be detected by low specific activity ^{125}I anti-immunoglobulin autoradiography (21). The presence of surface immunoglobulin on guinea pig lymphocytes also leads to their specific retention on anti-IgG coupled nylon plates (Fumio Takei - unpublished data). The effluent population from these plates are enriched in Ig-negative cells and the majority of Ig-bearing cells are removed. Similarly, but perhaps with less specificity, Ig-bearing cells are removed from a population of cells by passage through nylon fibre columns (38). Unfractionated cells and cell populations depleted of B cells using anti-Ig plates and nylon columns both contained antigen binding cells specific for the NH_2-terminal and COOH-terminal determinants, at least when measured using high specific activity antigens. The observation that the B-cell depleted population contains antigen binding cells suggests that these cells are antigen binding T lymphocytes. Several alternative explanations exist:

1) The antigen binding cells are B cells which escaped retention on the anti-IgG plates or nylon fibre columns. This is unlikely as 50% of the antigen binding B lymphocyte population would have to elute consistently in several experiments. Two classes of B cells may exist thus allowing selective retention of one of these B cell classes, although the B cell content varied markedly from experiment to experiment and makes this explanation difficult to believe.

2) Recently, it was shown by Webb and Cooper (136) that cytophilic

IgM antibody can adsorb to the surface of T cells. Thus it is possible that the lymphoid cells in an immune animal can bind antigen through acquired antibody. The failure to detect antibody to OFD in immunized guinea pigs (135), and the use of extensively washed cells in the work described here argues against binding via passively acquired antibody on T cells. It is possible, however, that acquired antibody on T cells is responsible for antigen binding as only very few molecules of antibody would be required. If, however, this were the case, it would not invalidate the observations of this work indicating the T cell function can be depressed specifically by inactivating binding cells.

3) The cells binding the antigenic determinants are T lymphocytes. This is the most likely explanation and is supported by the decreased T cell stimulation after specific antigen suicide on the B cell depleted populations.

Most reports of failure to detect T cells binding soluble antigen have involved the use of low specific activity radiolabelled molecules. Successful detection of antigen binding murine T lymphocytes was reported by Roelants (101) using high specific activity iodinated molecules and recently by Kelly et al (60) using the ferredoxin peptides coupled to poly D glutamic acid. It therefore appears that detection of antigen binding T cells depends upon the sensitivity of the assay system used, either because of small amounts of antigen bound by T lymphocytes or because of the transient nature of antigen binding, problems seemingly

overcome by the use of highly radioactive or multideterminant molecules with high binding avidity.

Lymphocyte stimulation results obtained with suicided cell populations indicated that specific inactivation of cells responding to OFD and $N_{10}C$ was achieved. The responses to the unrelated antigens KLH and PPD were not significantly reduced although some lowering of stimulation was seen in several experiments, possibly due to irradiation. The results from ^{125}I-sBSA treated cells are consistent with this idea. Successful antigen suicide was achieved with ^{125}I-$N_{10}C$ and with both antigenic determinants, although the NH_2-terminal conjugate was slightly more effective. The reason for this is not clear; perhaps slightly greater binding affinity of the NH_2-terminal accounts for the difference. Alternatively, the radiation from the NH_2-terminal determinant may be more effective as the ^{125}I label is part of the determinant itself whereas the COOH-terminal contains no tyrosine and the radioiodine is carried only in the s-BSA molecule.

Several problems were encountered in the suicide procedure:

1) Abrogation of the stimulation response was complete in only a few experiments. Autoradiography showed that a proportion of the determinant binding cells remained after antigen suicide. These cells may have responded to antigenic stimulation. Since the lymphocyte stimulation procedure utilizes sensitized lymphocytes, numbers of responding cells may be at different stages in the cell cycle and may escape inactivation either by binding insufficient antigen or by not binding antigen at all.

Progenitor development may occur over the period of assay, relatively unaffected by antigen suicide, and may respond to stimulation. Complete specific abrogation of antibody responses has been reported by several investigators (9, 132) although in all cases antigen suicide was performed on virgin (unsensitized) cell populations using adoptive transfer systems in inbred mice. A similar approach could be used in the work reported here using OFD (and synthetic peptide analogues) with inbred guinea pigs, an attack which was not carried out due to unavailability of these animals.

2) Scintillation counting of ^{3}H-thymidine incorporated into the DNA of responding lymphocytes was hindered by the low energy gamma emissions from bound ^{125}I-labelled molecules. This problem was partially solved by incubating the suicided cells in serum-free medium for 3 hours prior to washing and culturing. Cell surface proteins are shed during this incubation (127) and most of the bound ^{125}I-labelled antigen was removed. Interference from remaining ^{125}I was negated by subtracting counts from replicate cultures that had not been pulsed with ^{3}H-thymidine. Standard deviations between replicate samples were large, however, probably due to the high specific activity of the iodinated molecules. This may account for the great variation in the suicide results. Ideally, the DNA should be purified and counted, a procedure which was not followed here because of the technical burden involved in processing large

numbers of cell cultures.

Cell Cooperation

If lymphocytes are restricted to reacting with a single specificity of antigenic determinant, then the work of Waterfield et al (135) and Levy et al (64) suggests a two cell collaboration in the cell mediated response to the soluble protein antigen, OFD. The work in this thesis was designed to test this suggestion. Mixing of ^{125}I-NsBSA treated cultures with ^{125}I-CsBSA treated cultures resulted in a partial restoration of the stimulation response to OFD suggesting synergy between cells of the suicided cultures. Mixing experiments performed with B cell depleted cultures indicated that the observed synergy was between T lymphocytes. The response to KLH was not increased significantly in the same mixed populations, demonstrating that the observed synergy was specific for OFD stimulation. There are several possible explanations for these observations:

1) The increased stimulation may be due to priming of cells by residual (bound) N-sBSA and C-sBSA. Mixing of these cultures would result in a greater response to OFD than the cultures containing cells treated with only one determinant. This is an unlikely possibility, however, as the amount of bound peptide conjugate is insufficient to cause stimulation. The culture period is also too short to allow primary sensitization to the peptide conjugates.

2) The synergy is due to specific interaction between cells binding

the NH_2-terminal and COOH-terminal determinants, the mixed cultures containing viable cells of both types.

This second explanation is exciting as it is the first indication of T cell interaction to a soluble protein antigen. The confirmation of this work depends on positive demonstration of interaction using cell populations sensitized to each determinant separately in different inbred guinea pigs.

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