CELLULAR RESPONSES IN GUINEA PIGS SENSITIZED TO
1-FLUORO-2, 4-DINITROBENZENE

by

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I hereby recommend that this dissertation prepared under my direction by RONALD EDWARD PAQUE entitled "Cellular responses in guinea pigs sensitized to 1-fluoro-2,4-dinitrobenzene" be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy.

Dissertation Director 7/20/66

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ACKNOWLEDGMENT

The writer wishes to thank Dr. Wayburn S. Jeter for his help, patience, and guidance throughout this study. This work is dedicated to my parents, who have always encouraged my ambitions, and stimulated my academic pursuits.
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ABSTRACT

Lymphoid cells taken from guinea pigs sensitized to the chemical 2, 4-dinitrofluorobenzene increased in number when incubated in vitro with this haptene. The cellular increases noted in 30 experiments were approximately 46%, and these increases were evident after two hours of incubation, at 37 C.

The increase in cell numbers was not dependent on the presence of complement, was most dramatic when the animal was skin-tested, and appeared to possess immunologic specificity. The degree of cellular reactivity was optimum three weeks after sensitization and declined thereafter. Efforts to demonstrate mitosis of lymphocytes were unsuccessful. The mechanism(s) underlying the cellular increases was (were) unknown.
INTRODUCTION

In a number of diseases in which delayed type hypersensitivity played a dominant role, cellular damage resulted when cells from sensitive animals came in contact with antigen in vitro. One striking example of this was the fact that leucocytes from tuberculin sensitive animals were lysed in vitro when placed in contact with tuberculin and complement (9, 10, 25, 29). On the other hand, another study suggested that cellular proliferation occurred (30).

Thus, there was disagreement in the literature about the fate of tuberculin sensitive leucocytes incubated in vitro with tuberculin. Some of the manifestations described were cytotoxic phenomena (1, 16, 17, 25, 27), mitotic stimulation (2, 15, 18, 19, 30), inhibition of migration (8), and release of endogenous products from sensitive cells (3, 21). Other investigators reported leucocytic indifference to tuberculin (20). In a study involving sensitization to simple chemicals, Lubaroff and Ritts (22) reported that lymphocytes from guinea pigs sensitized to picryl chloride showed cytotoxicity when incubated in vitro with the haptene. This reaction was reported to be immunologically specific.

It is evident that much of the work concerning cellular reactions between leucocytes and antigens or haptenes in delayed-type
hypersensitivity states was of a contradictory nature. The majority of the investigations have employed tubercle bacilli or Bacilli-Calmette-Guérin (BCG) to induce a state of delayed hypersensitivity. It was recognized that tuberculin represents a mixture of various substances. Simple chemical compounds can also elicit a similar state of reactivity. With the exception of the work of Lubaroff and Ritts (22), and Holland and Maurer (19), little has been done to elucidate the reactions of cells from animals sensitized to simple chemicals, although these substances are much more homogenous than tuberculin.

The purpose of this study was to determine the effect of such a compound, 1-fluoro-2,4-dinitrobenzene, on cells from guinea pigs sensitized to this chemical.
MATERIALS AND METHODS

**Animals**

Animals employed in these experiments were guinea pigs of the Rockefeller strain, weighing approximately 350-650 g. Both male and female guinea pigs were used. They were fed Purina Guinea Pig Chow and the drinking water was fortified with Vitamin C. Cabbage was given daily as a dietary supplement.

**Chemical**

1-fluoro-2,4-dinitrobenzene (Eastman Organic Chemicals) was purified by recrystallization 3 times from absolute alcohol at -20 C. The crystals were transferred to a 50 ml flask and stored at room temperature, at which temperature the chemical is liquid.

**Sensitization of Guinea Pigs**

Guinea pigs were sensitized by topical application of 2,4-dintrofluorobenzene to a clipped area at the back of the neck of the animal. The area was approximately the size of a half dollar. Six drops of the chemical in a 2% alcoholic solution were applied daily for six consecutive days with a Pasteur pipette and rubbed on the shaved skin with a fire-polished glass rod (28).
Skin Testing

The animals were skin tested at various times from one week to four weeks after the last painting. Solutions of 0.1% and 0.5% of 2,4-dinitrofluorobenzene in olive oil were employed in the skin tests. One drop of each dilution of the chemical, and one drop of an olive oil control were applied to a shaved area on the back of the animal. A glass rod was utilized to spread the olive oil dilutions over an area of approximately one cm in diameter. The reactions were observed at 24 and 48 hours after skin testing.

Preparation of 2,4-dinitrofluorobenzene Conjugate

2,4-dinitrofluorobenzene conjugate was prepared according to the method of Porter and Sanger (26). Five hundred mg of serum protein, determined by the method of Gornall (12), was placed in a beaker with 100 ml of 0.15 M NaCl. Two-tenths ml of the chemical was added to the solution, and the pH adjusted to 8.0 with sodium bicarbonate, usually to saturation. The solution was incubated for 2 hours at room temperature and then placed at 4 C overnight. After overnight incubation, the solution was dialyzed against 0.15 M NaCl (20 volumes) at room temperature for a minimum of ten changes. The final volume of the conjugate was measured and sterilized by filtration through a Millipore filter having a pore size of 0.45 μ. The conjugate was stored in sterile serum bottles at 4 C.
Harvesting of Cells

After bleeding and sacrificing animals by heart puncture, the suprascapular lymph nodes were surgically removed from the guinea pigs. The nodes were quickly transferred to a petri dish containing approximately 15 ml of Hanks's balanced salt solution (14). Excess fat and muscle surrounding the lymph nodes were trimmed with small scissors. The excised nodes were washed several times in balanced salt solution, minced with scissors, and the cells expressed with a small spatula through a sterile 20 gauge stainless steel screen. The screen was rinsed with balanced salt solution over a small funnel draining into a 50 ml centrifuge tube. The resulting cellular suspension was centrifuged at 1800 rpm for 20 min on a No. 240 International centrifuge head.

Preparation of Cells for Incubation

After centrifugation of the expressed cells, the supernatant fluid was discarded and the cells were resuspended in 5-10 ml of fresh, homologous guinea pig serum, balanced salt solution, or serum which had been inactivated at 56 C for 30 min. One ml of cellular suspension was delivered to each tube (9x75 mm) containing 0.1 ml of 2, 4-dinitrofluorobenzene, 0.1 ml of conjugate, or 0.1 ml of balanced salt solution. The tubes were set up in duplicate.
The tubes were sealed with corks dipped in melted paraffin and held in place with masking tape to insure against spillage during incubation. The tubes were then incubated at 37 C for 2 hr in a rotating box at 2.5 rpm. Cotton was inserted tightly inside the rotating box to hold the tubes in place during incubation. After incubation, the tubes were removed and plunged in an ice bath prior to staining and counting the cells.

**White Cell Counts**

White cell counts were performed in duplicate on all cellular suspensions, and acetic acid was used as a diluent to lyse residual red blood cells present.

A pipette containing cells from each tube was put on a Bryan-Garry pipette rotor, Model PR-65, and rotated at least 10 min prior to counting. Throughout the counting period, all pipettes were rotated so that counts would represent randomly dispersed cellular suspensions (7). In addition, the Bryan-Garry pipette rotor insures that the cells in the diluting fluid within the pipette will follow a systematic chance distribution, and that any drop of fluid from the pipette is representative of its entire contents.

Each pipette was removed from the rotor at the appropriate time, and the first four to seven drops were discarded to insure that counts were made from the bulb of the pipette. Both counting
chambers of a Spencer Bright-Line hemacytometer were filled, and counts were performed using the 10X (16 mm) objective.

**Differential Counts**

Smears for microscopic examination were stained with Giemsa and Wright stain according to the method of Gurr (13), and examined under oil immersion. Differential counts were made by counting 100 cells, recording the cell types, and calculating on a percentage basis the quantity of various cell types.

**Vital Staining**

In addition to white cell counts, trypan blue dye exclusion tests were carried out to determine cell viability. A 0.5% solution of trypan blue in distilled water was prepared and filtered to remove any undissolved particles of dye.

The incubated cells were centrifuged at 1200 rpm for 8-12 min, the supernatant fluid discarded. The cells were then diluted with balanced salt solution to the original volume of one ml. A serological pipette was used to remove 0.2 ml of the resuspended cells to which 0.1 ml of the stain was added. Viable counts were made 5 to 10 minutes later. Cells taking up the blue stain are non-viable whereas viable cells exclude the dye.

In order to differentiate monocytes from lymphocytes, supravital staining was done on two slides prepared from each tube.
used in the experiment. A .25% solution of Vital Neutral Red, 1.75 ml, was mixed with .07 ml of a .4% solution of Vital Jonas Green in 10 ml of absolute alcohol (13).

The stain was flooded on clean glass slides, whereupon the slides were air dried, and stored. Two hundred cells were counted on each slide with monocytes showing yellow to light orange granules. Lymphocytes, on the other hand, have no granules.

**Specificity Studies**

Specificity studies were carried out on cells from animals sensitized to 2,4-dinitrofluorobenzene with analogous and non-analogous compounds. The procedure for preparation and incubation of cells was essentially the same as previously described with the following exception. 0.1 ml of citraconic anhydride (Eastman Organic Chemicals), and 0.1 ml of a .04% solution of O-dinitrobenzene (Mallinkrodt Co.) were incubated with the sensitized cells instead of 2,4-dinitrofluorobenzene. Counts and staining were performed in the manner previously described.

**Colchicine Studies**

Studies were performed incorporating colchicine into the systems along with 2,4-dinitrofluorobenzene in an effort to halt possible mitosis. The procedure was essentially the same as previously described, but with a 0.1 ml solution of colchicine
containing 1.0 μg being added to the various tubes. After two hours of incubation, smears, stains, and counts were done in the usual manner.
EXPERIMENTAL RESULTS

Preliminary studies with blood and peritoneal exudative cells showed that 2,4-dinitrofluorobenzene stimulates the proliferation of cells from sensitized guinea pigs in some cases, and decrease the number in others. The percent change observed in 9 experiments was from +14.6 to +96.4% increase over control tubes. In four other experiments, the average percent change was from -7.7 to -25.8%. In one experiment in which 2,4-dinitrofluorobenzene was conjugated to guinea pig red blood cells (6), an increase of +54.7% was observed. Cell increases observed in these studies were not affected by the presence or absence of either complement or serum.

In an attempt to achieve a more uniform cell population, later experiments were confined to cells expressed from lymph nodes. Differential counts on such nodal cells revealed populations of 92-98% small lymphocytes, and 2-8% large mononuclear cells. In ten experiments, free 2,4-dinitrofluorobenzene was incubated with lymph node cells from sensitized guinea pigs. In nine of these, total cell increase ranged from +9.8 to 99% (Table 1). In ten experiments in which the conjugate was incubated with the cells, experiments 1, 4, 5, 7, and 9 had increases of the cell population, whereas in three experiments, slight decreases were observed (Table 1).
TABLE I

WHITE CELL COUNTS ON LYMPH NODE PREPARATIONS FROM GUINEA PIGS SENSITIZED TO 2, 4-DINITROFLUOROBENZENE*

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Initial $^{a}$</th>
<th>Control $^{b}$</th>
<th>Conjugate $^{c}$</th>
<th>2, 4 DNB $^{d}$</th>
<th>% Change Conjugate $^{e}$</th>
<th>% Change Chemical $^{f}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22,900+</td>
<td>19,650</td>
<td>21,000</td>
<td></td>
<td>+16.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12,650</td>
<td>12,950</td>
<td>12,650</td>
<td>18,050</td>
<td>-2.3</td>
<td>+39.4</td>
</tr>
<tr>
<td>3</td>
<td>7,400</td>
<td>7,300</td>
<td>6,400</td>
<td>12,400</td>
<td>-12.3</td>
<td>+69.9</td>
</tr>
<tr>
<td>4</td>
<td>13,000</td>
<td>12,700</td>
<td>15,200</td>
<td>13,950</td>
<td>+19.7</td>
<td>+9.8</td>
</tr>
<tr>
<td>5</td>
<td>14,450</td>
<td>14,800</td>
<td>17,300</td>
<td>29,450</td>
<td>+16.9</td>
<td>+99.0</td>
</tr>
<tr>
<td>6</td>
<td>15,050</td>
<td>14,700</td>
<td></td>
<td>26,250</td>
<td></td>
<td>+78.6</td>
</tr>
<tr>
<td>7</td>
<td>11,000</td>
<td>10,950</td>
<td>11,150</td>
<td>20,200</td>
<td>+1.8</td>
<td>+84.5</td>
</tr>
<tr>
<td>8</td>
<td>31,350</td>
<td>30,950</td>
<td></td>
<td>38,250</td>
<td></td>
<td>+23.6</td>
</tr>
<tr>
<td>9</td>
<td>20,150</td>
<td>20,200</td>
<td>20,350</td>
<td>28,350</td>
<td>+0.7</td>
<td>+40.3</td>
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<tr>
<td>10</td>
<td>11,550</td>
<td>11,450</td>
<td>10,650</td>
<td>14,400</td>
<td>-7.0</td>
<td>+25.8</td>
</tr>
</tbody>
</table>

$^{a}$ Initial count on cellular suspension
$^{b}$ Control count--cells incubated with BSS
$^{c}$ Count on cells incubated with conjugate
$^{d}$ Count on cells incubated with free chemical
$^{e}$ Per cent change from control count-conjugate
$^{f}$ Per cent change from control count-chemical
$^{+}$ WBC's/cubic mm
$^{*}$ Incubation time: 120 min
$^{g}$ Student t analysis of paired experiments indicate a confidence level of 99.0%
In 21 experiments performed, cell increases of +9.6 to 99% were noted when lymph node cells from sensitized animals were incubated with 2,4-dinitrofluorobenzene. The mean per cent change for all experiments was +45.5% increase.

The data from seven complete experiments from Table I were subjected to Student's t test (4) for paired experiments in order to determine the level of confidence. It was found that the mean increase of approximately 50% for these experiments had a confidence level slightly in excess of 99.0%.

This proves that the average increases in the number of lymphocytes of approximately 50%, observed with cells from sensitized animals, when incubated with 2,4-dinitrofluorobenzene, are with 99% probability and not due to chance.

A series of experiments were done in which lymph node cells from non-sensitized animals were tested (Table II). Increase of the normal total cell population with 2,4-dinitrofluorobenzene was observed. The increases ranged from +1.5 to +13.5% with a mean of +6.08%, and were not as dramatic as those described from sensitive animals (Table II). The results obtained with the conjugate were equivocal. Slight decreases of normal cell numbers in some experiments were evident (-1.3 to -16.5%), whereas small increases of +4.5% and +4.9% were noted in others (Table II).
### TABLE II

WHITE CELL COUNTS ON LYMPH NODE PREPARATIONS FROM NON-SENSITIZED GUINEA PIGS*

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Initial(^a)</th>
<th>Control(^b)</th>
<th>Conjugate(^c)</th>
<th>2,4 DNFB(^d)</th>
<th>% Change Conjugate(^e)</th>
<th>% Change Chemical(^f)</th>
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<tr>
<td>11</td>
<td>13,400 (^+)</td>
<td>13,000</td>
<td>12,750</td>
<td>13,750</td>
<td>-1.9</td>
<td>+5.8</td>
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<tr>
<td>12</td>
<td>9,600</td>
<td>5,550</td>
<td>5,800</td>
<td>6,300</td>
<td>+4.5</td>
<td>+13.5</td>
</tr>
<tr>
<td>13</td>
<td>15,700</td>
<td>15,250</td>
<td>16,000</td>
<td>15,700</td>
<td>+4.9</td>
<td>+3.0</td>
</tr>
<tr>
<td>14</td>
<td>27,300</td>
<td>26,150</td>
<td>25,800</td>
<td>26,550</td>
<td>-1.3</td>
<td>+1.5</td>
</tr>
<tr>
<td>15</td>
<td>25,450</td>
<td>24,300</td>
<td>20,300</td>
<td>25,900</td>
<td>-16.5</td>
<td>+6.6</td>
</tr>
</tbody>
</table>

\(^a\) Initial count on cellular suspension  
\(^b\) Control count--cells incubated with BSS  
\(^c\) Count on cells incubated with conjugate  
\(^d\) Count on cells incubated with free chemical  
\(^e\) Per cent change from control count-conjugate  
\(^f\) Per cent change from control count-chemical  
\(^+\) WBC's/ cubic mm  
* Incubation time: 120 min
Experiments were performed to determine the duration of the responsiveness to 2, 4-dinitrofluorobenzene of cells from sensitized guinea pigs. These tests were done 1-4 weeks after sensitization. The results are presented in Table III. The mean percent increase after one week was +38.0%, two weeks +48.5%, three weeks +52.5%, and after four weeks, +26.2% (Fig. 1).

The maximum percentage increase was evident three weeks after the last application of the chemical. Three week maximum cell reactivity and increase was consistent with the earlier work done with tuberculin (30). After four weeks, it appeared that cell increases were not as great as observed earlier, declining to +26.2%. Yet a +49.4% increase was noted in one of the experiments done after four weeks, and increases of only +25.8% and +19.3% were evident at one and two weeks after painting. These results were inconsistent. The most consistent and notable cell increases were observed three weeks after the last chemical application.

Skin-testing the animal 24 hours before harvesting lymph node cells enhanced the increase effect. In experiments 3, 5, 6, and 7, where animals were skin-tested, increases of +69.9%, +99.0%, +78.6% and +84.5% respectively are shown (Table I). Normal animals were also tested, no skin reactivity or heightening of the response was observed.
<table>
<thead>
<tr>
<th>Time after Sensitization</th>
<th>Initial</th>
<th>Control</th>
<th>Conjugate</th>
<th>2, 4 DNFB</th>
<th>% Change</th>
<th>Conjugate</th>
<th>Chemical</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>20,150</td>
<td>20,150</td>
<td>20,350</td>
<td>28,350</td>
<td>-0.7</td>
<td>+40.7</td>
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<tr>
<td>1 week</td>
<td>11,550</td>
<td>11,450</td>
<td>10,650</td>
<td>14,400</td>
<td>-6.9</td>
<td>+25.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10,550</td>
<td>10,450</td>
<td>10,100</td>
<td>15,400</td>
<td>-3.3</td>
<td>+47.4</td>
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</tr>
<tr>
<td></td>
<td>9,550</td>
<td>9,350</td>
<td>11,000</td>
<td>13,200</td>
<td>+17.6</td>
<td>+41.2</td>
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<tr>
<td>2 weeks</td>
<td>10,600</td>
<td>10,950</td>
<td>11,200</td>
<td>20,250</td>
<td>+2.3</td>
<td>+84.9</td>
<td></td>
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<tr>
<td></td>
<td>13,000</td>
<td>12,700</td>
<td>15,200</td>
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<td>+19.7</td>
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<td></td>
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<td>-2.3</td>
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<td>7,300</td>
<td>6,400</td>
<td>12,400</td>
<td>-12.3</td>
<td>+69.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8,250</td>
<td>8,450</td>
<td>8,750</td>
<td>11,650</td>
<td>+3.5</td>
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<td>12,800</td>
<td>14,550</td>
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<td>+49.4</td>
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<tr>
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<td>6,000</td>
<td>6,450</td>
<td>+6.2</td>
<td>+14.2</td>
<td></td>
</tr>
</tbody>
</table>

* "Time of collection of cells after last day of painting"
Fig. 1. Effect of time after sensitization on susceptibility of lymph node cells to action 2, 4-dinitrofluorobenzene.

+ Mean per cent increase of three experiments.

* Range of counts.
In order to determine whether complement was a factor necessary for cellular increase, cells were suspended in fresh, homologous guinea pig serum and serum which had been inactivated at 56°C for 30 min. Increases in cell numbers were not as great in inactivated serum as in those observed in fresh serum. Experiments numbered 8, 17, 21, 24, and 27 were done in serum that had been inactivated at 56°C for 30 min (Tables I and III). The mean per cent increase for these experiments was +24.1%. Consistently greater increases were noted with fresh serum (complement present), which was used in experiments 2, 3, 6, 7, and 9 (Table I).

Differential counts were performed on all incubated specimens. Smears stained with both Wright and Giemsa stain revealed a uniform population of small lymphocytes. The percentage of lymphocytes was always above 90.0%. Monocytes were differentiated from lymphocytes by supra-vital staining on a warm stage at 37°C. Very few monocytes were seen, rarely in excess of one or two per cent and usually less than one per cent. Occasionally unidentified large cells were seen in smears from control and experimental tubes in quantities of two to seven per cent.

Trypan blue dye exclusion test usually revealed a uniform population of viable lymphocytes. Rarely were non-viable cells seen in the various preparations examined.
The total number of cells used in the various experimental systems during incubation ranged from approximately 6000 cells/cubic mm to 26000 cells/cubic mm. In systems where 2,4-dinitrofluorobenzene was incubated with sensitized cells, total cell increases ranged from approximately +800 cells/cubic mm to nearly +15,000 cells/cubic mm. Normal cell increases with the chemical ranged from +350 cells/cubic mm to +1600 cells/cubic mm.

The total numbers of sensitized cell increases noted with the conjugate were from +150 cells/cubic mm to +2500 cells/cubic mm. Total decreases in sensitized cell numbers with the conjugate ranged from -350 cells/cubic mm to nearly -1000 cells/cubic mm, however most decreases were from -800 to -900 cells/cubic mm. The normal cell decreases noted with conjugate were approximately -350 cells/cubic mm, and increases in normal cell numbers of +250 cells/cubic mm to +750 cells/cubic mm.

A timed study was done in order to plot the rate of cellular increase over a 2 hr period. The results are shown in Fig. 2. After 15 min a sharp cellular increase was evident, which remained fairly constant up to 30 min. After 30 min another cellular increase was observed, which declined slightly at the end of 2 hr.

Five experiments were performed in which citraconic anhydride was used as a heterologous chemical in order to test the specificity of the effect. The results are shown in Table IV. In
Fig. 2. Numbers of cells after incubation with 2, 4-dinitrofluorobenzene at 37 C.
TABLE IV

WHITE CELL COUNTS ON LYMPH NODE PREPARATIONS FROM GUINEA PIGS SENSITIZED TO 2, 4-DINITROFLUOROBENZENE AND INCUBATED WITH CITRACONIC ANHYDRIDE FOR 2 HOURS AT 37 C

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Initial</th>
<th>Control</th>
<th>Citraconic anhydride</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>9,600</td>
<td>9,700</td>
<td>9,700</td>
<td>0.0</td>
</tr>
<tr>
<td>32</td>
<td>6,350</td>
<td>6,200</td>
<td>6,450</td>
<td>+4.0</td>
</tr>
<tr>
<td>33</td>
<td>8,950</td>
<td>9,100</td>
<td>8,950</td>
<td>-1.6</td>
</tr>
<tr>
<td>34</td>
<td>11,250</td>
<td>10,750</td>
<td>10,900</td>
<td>+1.3</td>
</tr>
<tr>
<td>35</td>
<td>10,300</td>
<td>9,900</td>
<td>10,150</td>
<td>+2.1</td>
</tr>
</tbody>
</table>

+WBC's/cubic mm
three experiments, slight increases of 1.3%, 2.1%, and 4.0% were noted. One experiment showed a slight decrease in cell numbers amounting to -1.3%. The remaining experiment exhibited neither increase nor decrease of cell numbers using citraconic anhydride. The results of five experiments using a .04% solution of 0-dinitrobenzene were essentially the same as those observed with citraconic anhydride. These results are shown in Table V.

One attempt was made to count cells on the Coulter counter. It was impossible to get an accurate cell count due to the presence of extracellular debris.
TABLE V
WHITE CELL COUNTS ON LYMPH NODE PREPARATIONS FROM
GUINEA PIGS SENSITIZED TO 2, 4-DINITROFLUOROBENZENE
AND INCUBATED WITH 0-DINITROBENZENE
FOR 2 HOURS AT 37 C

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>Initial</th>
<th>Control</th>
<th>0-Dinitrobenzene</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>12,850</td>
<td>12,500</td>
<td>12,450</td>
<td>0.0</td>
</tr>
<tr>
<td>37</td>
<td>5,750</td>
<td>6,050</td>
<td>6,450</td>
<td>+6.9</td>
</tr>
<tr>
<td>38</td>
<td>9,350</td>
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</tr>
<tr>
<td>39</td>
<td>8,150</td>
<td>7,950</td>
<td>7,850</td>
<td>-1.5</td>
</tr>
<tr>
<td>40</td>
<td>7,750</td>
<td>7,000</td>
<td>7,650</td>
<td>+9.0</td>
</tr>
</tbody>
</table>

+WBC's / cubic mm
DISCUSSION

Mitosis of lymphocytes from animals sensitized to tuberculin has been shown by several workers (18, 19, 30). Other investigators reported cytolysis (9, 23), cytotoxic effects (17, 24), and lymphocytic indifference (5, 20) to tuberculin. Since tuberculin represents a mixture of many substances, it seemed appropriate to test the effect of a simple, organic compound on cells from animals sensitized to the compound. A chemical of this sort is less complex, and much more uniform chemically.

Recently, Lubaroff and Ritts (22) described the specific cytotoxic action of picryl chloride, another simple organic compound, on lymphocytes from guinea pigs sensitized to the chemical. The results of this study were in disagreement with those of Lubaroff and Ritts (22).

It appeared that lymphocytes from guinea pigs sensitized to 2,4-dinitrofluorobenzene undergo mitosis when incubated with the chemical in vitro. These results were in agreement with Holland and Maurer (19) who found that sensitized lymphocytes from human beings undergo mitosis in vitro. The reproducible increases of cells in experimental systems having sensitized cells and chemical indicated that mitosis had taken place during the two hour incubation period.
It was tempting to postulate that lymphocytes from sensitized animals reacted to the chemical by undergoing mitosis as a manifestation of the immune reaction. If so, this study lends support to Burnet's clonal selection theory, where sensitized lymphocytes upon "recognizing" 2, 4-dinitrofluorobenzene as the sensitizer, multiply, and produce antibody in response to the haptene which has helped to induce the state of reactivity. Although mitosis has not been shown, it was interesting to speculate on the origin of the apparent cell increases observed in tubes with 2, 4-dinitrofluorobenzene present. The increase seemed to be most dramatic when cells from animals sensitized for two to three weeks were used. This optimum reactivity at three weeks was consistent with mitosis observed in earlier work done with tuberculin (30).

Cellular increase was even more dramatic when animals were skin tested 24-48 hours before cell harvest. Perhaps skin testing "primes" the already sensitized lymphocytes so they react even more vigorously when brought in contact with the sensitizing chemical. This result represents a sort of cellular anamestic response in vitro.

Some studies have shown that lymphocytes undergo morphological transition to a blast-like cell after undergoing mitosis (15, 19, 30). In this study, lymphocytic transformation was not observed. On the other hand, it was purely speculative as to whether lymphocytic transformation was a necessary corollary to lymphocytic mitosis. The
results here indicated that if mitosis does in fact occur with 2,4-dinitrofluorobenzene, lymphocytic transformation does not take place, since microscopic examination of lymphocytes reveals little evidence of large, blast-like cells.

Holland and Maurer (19) failed to show mitosis of human lymphocytes from an individual sensitized to sodium diphenylhydantoin, but they were able to show an increase in lymphocyte populations when sensitized lymphocytes were incubated with phenytoin. Lymphocytic transformation was also observed.

From the data presented here, it appears that 2,4-dinitrofluorobenzene exerted a stimulating effect on sensitized cells causing them to multiply. The inconsistent results obtained when cells were incubated with conjugate were difficult to explain. In some experiments, cell increases were observed, whereas in others, cell decreases were seen. It would seem these increases and decreases were a reflection of the availability of the haptene within the conjugate. Coupling 2,4-dinitrofluorobenzene to serum protein changes the chemical character of the haptene and consequently less haptene may be available to stimulate the sensitized cell. The homogeneity of haptene conjugates has not been determined, but perhaps sensitized cell populations react in varying degrees to the presence or absence of haptene; a manifestation of the spectrum of immune response.
It was apparent that a variety of responses was evident when cells sensitized to tuberculin were incubated with this substance. Considering the work of Lubaroff and Ritts (22), Holland and Maurer (19), and the present study, it appeared the experiments with simple organic chemicals imitate the variety of results obtained with tuberculin. The disparity of results observed with tuberculin and simple organic chemicals suggested an inherent divergency of immune responses of sensitized cells and animals rather than chemical variations.

The increase effect was independent of complement. Although various investigators have found complement to be necessary for cytolysis and cytotoxic effects (9, 17), the presence of complement appeared not to be necessary for cellular increase (29). Results obtained when inactivated serum was used were not much different from those obtained in fresh, homologous serum. Since there were many manifestations of the immune response in vitro which depend on the presence of complement, these results suggest that the cellular increase does not depend on complement.

The increases were specific. Cells taken from animals sensitized to 2,4-dinitrofluorobenzene did not exhibit increases when incubated with two different heterologous chemicals. Utilizing 0-dinitrobenzene, an analogue of 2,4-dinitrofluorobenzene, lymphocytic increases were not observed when incubated with 2,4-dinitrofluorobenzene
sensitized cells. Citraconic anhydride, a non-analogous chemical, also failed to induce cellular increases. These results suggested a true immunologic specificity of the increase.

It was tempting to speculate that 2,4-dinitrofluorobenzene, acting as a haptene, can confer cellular specificity on lymphocytes in sensitized animals. Also, it appears that this specificity and reactivity is different from that induced by picryl chloride, where a cytotoxic effect on lymphocytes was evident (22). The cytotoxic effect with picryl chloride occurred after two hours.

Sometimes clumping of cellular populations was evident in systems where chemicals and serum were incubated with cells. However, clumping was usually absent in our tests, and all glassware was siliconized which helped reduce cellular adherence to the walls of the test tubes. Consequently, the counts probably represented a fairly true picture of the cell numbers present in the systems. If increase in cell numbers was in reality an artifact and nonspecific, then it seems the use of a chemical analogue would show similar increases in cell populations. Such was not the case. Even the use of the homologous haptene conjugate did not produce the consistent and dramatic increases evident with free 2,4-dinitrofluorobenzene.

The most dramatic result evident from these data was the short period of time required for cellular increases. Mitosis of
lymphocytes has been reported to require at least 12-72 hours before increase in cell populations were detected; even when phytohemagglutinin was incorporated into the growth medium. When counts were made immediately before incubation, there appeared to be no cellular increases. Yet approximately 30-60 min later, cellular increases were evident. This suggested an almost synchronous growth pattern. Attempts to see these cells in the midst of their division have been unsuccessful. Colchicine has been incorporated into the systems in an effort to halt the cells in the spindle phase, whereby chromosome staining would reveal mitotic figures. These experiments have proved to be unsuccessful.

It was possible to make several conclusions about the data presented. First, it was established that when sensitized lymph node cells were incubated with the haptene, there were more cells present in tubes containing chemical, as compared to control tubes incubated with balanced salt solution. This was true even when the initial quantity of cells in all systems was virtually the same. Second, the apparent increased cell numbers were evident after only 120 min incubation, and the presence of complement was not required. Next, the increase effect appeared to be specific, since one chemical analogue and a non-analogous chemical could not duplicate the increase effect observed with 2,4-dinitrofluorobenzene.
One cannot say that these cells were actually undergoing mitosis because mitosis has not been observed or demonstrated. The techniques utilized to determine whether the cells were undergoing mitosis might not have been adequate. Consequently, more work would be required to determine exactly what happened to sensitized lymphocytes observed in the presence of 2,4-dinitrofluorobenzene.
SUMMARY

The effect of 2, 4-dinitrofluorobenzene on lymph node cells from sensitized guinea pigs has been determined.

Populations of sensitized lymph node cells increased in numbers when incubated with the homologous haptene, 2, 4-dinitrofluorobenzene, although cells incubated with serum conjugate exhibited decreases as well as slight increases.

Cells from non-sensitized animals also exhibited consistently slight increases with 2, 4-dinitrofluorobenzene. These increases were within a 10% experimental error incurred during counting, and were not nearly as dramatic as increased cell numbers from sensitized animals.

Early pilot studies on mixed populations of peritoneal exudative cells and cells from whole blood exhibited inconsistent results, with decreases and increases of the total cell population being evident.

The increase was independent of complement.

Skin testing the animal 24-48 hours before removing lymph node cells appeared to augment the effect, and after four weeks a lessening of the effect was seemingly evident.
Mitosis of sensitized lymphocytes was not demonstrated, and there was no conversion to a blast-like cell type. The mechanism underlying the increase was unknown.
LIST OF REFERENCES


