COMPUTER ANALYSES OF LYMPHOCYTE ALTERATIONS INDUCED BY IMMUNOSUPPRESSIVE CHEMICAL AGENTS

by

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I hereby recommend that this dissertation prepared under my direction by Preston Harold McKee

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After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

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SIGNED: Preston H. McKee

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ABSTRACT

Changes in guinea pig peripheral blood lymphocytes caused by the immunosuppressant chemicals 5-Flourouracil (5 FU) and cyclophosphamide and the sensitizing agent 1-chloro-2,4-dinitrobenzene (DNCB) were studied by scanning microphotometry and computer analyses of digitized cell images. Total and differential peripheral white blood cell counts were also made.

Parameters studied included optical density of Feulgen stained nuclei, nuclear area, granularity of chromatin, and chromatin distribution patterns.

Groups of outbred guinea pigs were injected intraperitoneally with 5 FU or cyclophosphamide or painted topically with DNCB. In some experiments 5 FU injections and DNCB applications were combined.

Topical application of DNCB resulted in a significant change in lymphocyte nuclei. Optical density, chromatin granularity, and nuclear area all showed a similar response of initial decrease, followed by a return to near initial values followed by another decline over the course of an experiment. Injection of 5 FU with DNCB treatment completely inhibited this response. Injection of 5 FU alone resulted in a marked decrease in nuclear optical density. Cyclophosphamide treatment resulted in no significant change in optical density but the chromatin distribution pattern of most lymphocytes underwent a shift toward lower optical density profiles. Peripheral blood lymphocyte levels were lower

at the conclusion of experiments than initially with the exception that DNCB application resulted in no discernible pattern.

INTRODUCTION

The target cell of the action of immunosuppressant chemicals is the lymphocyte (1,2). It has been firmly established that the lymphocyte is an immunocompetent cell. This is true of both immune responses transferred to another animal by means of antibody containing serum from a sensitized donor (humoral immunity) and those transferred by sensitized cells but not serum, (cell-mediated immunity). Lymphocytes from the thoracic duct of sensitized animals can transfer both cellular and humoral immunity to irradiated animals, and thoracic duct drainage of lymphocytes can impair the immune response (3).

It also appears that two different types of lymphocytes are responsible for humoral and cell mediated immunity (4). One type requires the thymus gland for development and these are called T cells. They are principally responsible for cell mediated immunity in which the immune reaction is mediated through cell-bound antibody and certain soluble factors released by the lymphocyte. Such reactions include delayed type sensitivity to simple chemicals such as 1-chloro-2,4-dinitrobenzene (DNCB), host rejection of transplanted foreign tissue, and cellular resistance to certain microorganisms such as the mycobacteria. The other type of lymphocytes mediating humoral responses such as circulating antibody formation and immediate sensitivity develop independent of the thymus. They are called B cells.

In birds, removal of the Bursa of Fabricus results in loss of humoral immunity, while in birds and other animals neonatal thymectomy

impairs cellular immunity with antibody levels remaining near those in control animals (5).

Further evidence for the two distinct lymphoid populations is derived from immunological deficiency diseases (4). Patients with Bruton-type congenital agammaglobulinaemia can not make antibody and are deficient in lymphoid cells producing antibody, but they have normal cell-mediated immunity. Children with congenitally hypoplastic thymus glands, as in Dr. George's syndrome, have greatly impaired cell-mediated immunity but can make almost normal amounts of antibody to some antigens.

There is also evidence, however, that these two lymphoid populations cooperate in some humoral immune reactions with the T cell acting as a "helper" for the B cell to process non-repetitive nonlinear spaced antigens (6). In irradiated animals, hemolysin production and antibody response to foreign serum proteins was much higher when both cell types were injected into the animals than either alone (7-10).

While T and B cells are morphologically indistinguishable they can be differentiated by different properties they possess and their response to physical and chemical agents.

They have different surface antigens, for example, mouse T cells have a 0 antigen and the B cells a B lymphocyte antigen. Thus, antisera specific for either cell type can be made (4). B cells can bind antibody-antigen-complement complexes by surface complement receptors (11) and antibody-antigen complexes by means of receptors for the Fc part of complexed Ig (12). T cells have a longer average generation

time (13), are slightly larger (14), are more dense and less adherent to materials (11), and are more negatively charged than B cells (15). Some mitogens such as concanavalin A, phytohaemaglutinin, and lentil stimulate only T cells, whereas lipopolysaccharides and anti-Ig sera stimulate only B cells (16). T lymphocytes are less susceptible to cytotoxic drugs, such as cyclophosphamide (17), corticosteroids (18), and irradiation (19) than B cells are. T lymphocytes possess less surface immunoglobulin than B cells do.

T and B cells are found in somewhat separate areas (20, 21). cells predominate in the periarteriolar sheath of the spleen, paracortex of lymph nodes, and interfollicular areas of gastrointestinal lymphoid tissues. B cells predominate in lymph follicles and peripheral regions of splenic white pulp, follicles and medulla of lymph nodes, and follicles of gastrointestinal lymphoid tissue. When radio labeled T and B cells are injected into an animal they migrate specifically to their respective areas.

Most T lymphocytes continuously recirculate between the blood and lymph, passing out of the blood through specialized post-capillary venules in the lymph nodes and Peyers patches, passing through the substance of the lymphoid tissue, enter the efferent lymph, and then reentering the bloodstream by the thoracic duct. Because they are the predominate circulating lymphocyte they are more susceptible to anti-lymphocytic serum than B cells are. Most B lymphocytes don't recirculate, although a few do, but through different areas of lymphoid tissue and with a slower transit time than T cells (22).

T and B cells also differ markedly in their transformation in response to antigenic stimulation (4). When B cells are activated by antigen they divide and differentiate into blast cells with abundant endoplasmic reticulum, and some develop into plasma cells. These cells remain in the lymphoid tissue and secrete large amounts of antibody which circulates in the blood.

When T cells are activated by antigen they proliferate and differentiate to become blast cells, but they do not develop significant endoplasmic reticulum and do not become antibody-secreting cells. They do secrete non-antigen specific factors including migration inhibition factor, chemotactic factors, cytotoxic factors, and mitogenic factors.

Immunosuppressant chemicals have been reported to affect both cellular and humoral immune response, although the degree of immunosuppression achieved depends on a number of variables, including the dose, time of injection, chemical used, and type of immunity being considered (23-35).

Immunosuppressant chemicals are thought to lower the immune response through a number of different mechanisms. Alkylating agents such as cyclophosphamide bind to guanine groups of DNA, causing cross-linking of nucleic acid strands and thus interfering with replication of nucleic acids and synthesis of proteins and enzymes (23). The agent 5-flourouracil (5 FU) is incorporated into nucleic acids replacing uracil (36). It may also act by inhibiting thymidylate synthetase thus depleting thymidine (37). Azathioprine, which is converted into 6-mercaptopurine (6 MP) <u>in vivo</u> (38) inhibits the conversion of inosinic

acid to xanthylic acid or adenylic acid, thus interfering with DNA replication. Incorporation of thiosinic acid derived from 6 MP into DNA or RNA leads to incorrect templates (39).

Azathioprine is not as effective an immunosuppressant in the guinea pig as in other animals (31). The agent 5 FU has not been found to be effective in animals, including the guinea pig (26), but it has in humans (34). Cyclophosphamide has a potent immunosuppressive effect in guinea pigs in delayed hypersensitivity reactions (40, 41).

Delayed hypersensitivity may develop toward simple chemicals such as 1-chloro-2,4-dinitrobenzene (DNCB) conjugated to protein carriers (42). As in other forms of delayed reactivity, the cell-antigen interaction leads to a morphologic and functional transformation of immunocompetent cells (42). The immunosuppressant 6-MP was found to inhibit skin test reactivity to tuberculin in guinea pigs for up to six weeks (43). Such reactivity would normally result macroscopically in an intense, red inflammatory reaction upon topical application of the sensitizing agent to a sensitized animal (44). As characteristic of delayed reactions, this response would not become evident for at least eight hours and reach a maximum between 24 and 48 hours.

Scanning ultraviolet microphotometry and computer analysis of digitized cell images have been used to study lymphocytes (45). Bartels et al. (46) analyzed human lymphocytes and Kiehn (47) analyzed guinea pig lymphocytes undergoing transformation in vitro in response to phytohemaglutinin. Jarkowski et al. (48) investigated alterations caused by lymphocytic leukemia in human lymphocytes. Olson et al. found

no large differences in large lymphocytes from the bursa and thymus of the chicken but found thymic small lymphocytes were slightly larger in nuclear area and more densely stained with Feulgen stain than bursal small lymphocytes by the seventh postnatal day (49). Bartels, Jeter, and Cole (50) found that 5 FUdR caused an increase in the nuclear area and a decrease in nuclear density in guinea pig lymphocytes. Scanning microphotometry and computer analysis of digitized cell images have not been used to study the effects of immunosuppressant chemicals on guinea pig lymphocytes in vivo except for FUdR or effects resulting from DNCB sensitization alone or in combination with immunosuppressant chemicals.

The purpose of this investigation was to detect changes by image analyses in guinea pig peripheral blood lymphocytes caused by immunosuppressant chemicals or the sensitizing agent 2,4 DNCB. In addition, the combination of DNCB with one of the chemicals, 5 FU was studied.

MATERIALS AND METHODS

Experimental Animals

Outbred Rockefeller and Amana strain albino guinea pigs of both sexes were used. The animals were obtained from colonies of the Department of Microbiology, The University of Arizona. The age of the animals varied from about six months to a year. Their weight ranged from 400 to 900 grams. They were individually housed in stainless steel cages. They were maintained in Purina guinea pig chow and water containing 0.3% ascorbic acid ad libitum. In addition, the diet included fresh cabbage given daily.

Immunosuppressing Agents

Solutions of 5-fluorouracil, azathioprine and cyclophosphamide in saline were used as the immunosuppressing agents. A solution of 2 mg. azathioprine in one ml. 0.15 molar NaCl was injected intra-peritoneally twice daily eight hours between injections every day except the last day of an experiment. A solution of 5 mg. cyclophosphamide in 2 ml. saline was injected intra-peritoneally daily every day except the last day of an experiment. Three different concentrations of 5-fluorouracil were injected intra-muscularly. These included a solution of 37.5 mg. of the chemical in 3.75 ml. injected twice daily six hours between injections, a solution of 37.5 mg. of the chemical in 0.75 ml. given twice daily three hours between injections, and a solution of 15 mg. of the chemical in 0.3 ml. saline given daily.

Sensitizing Agent

A 2% solution of 1-chloro-2,4-dinitrobenzene (DNCB) in 95% ethyl alcohol was used as the sensitizing agent in all experiments. The chemical was applied by dropper to an area 3 cm. in diameter on the nape of the neck which had first been clipped free of hair. It was spread with a fire-polished glass rod. Guinea pigs to be sensitized received topical application of 5 drops of 2% DNCB applied daily for six days to the same site.

Anaesthesia

Ether was used as the anaesthetic agent. It was applied to cotton in a small container which was held over the animal's head until it was unconscious.

Lymphocyte Collection

A volume of approximately 1 ml. of peripheral blood was extracted from the heart. Lymphocytes were separated by two methods. The first method was to centrifuge whole blood in test tubes at 1500 rpm, remove the buffy coat and plasma and recentrifuge twice in Wintrobe tubes with saline. The second method was to overlay 0.5 ml. whole blood into test tubes containing 1 ml. isopaque-methyl cellulose, centrifuge at 500 rpm for 25 minutes, extract the upper third of the isopaque-methyl cellulose containing the lymphocytes, and resuspend and recentrifuge the lymphocytes twice in saline (1). Using either method, the cells were resuspended in a few drops of saline, added to slides and

spread on the slides by loop or another slide. They were then stained by Schiff's reagent, a nucleophilic staining agent (50).

Staining

The lymphocytes were fixed and hydrated through ethanol washes of 95%, 70%, 50%, and 30% for one minute each. Slides were then hydrolyzed in 5 N HCl for 20 minutes.

After hydrolysis cells were rinsed in 1 N HCl and stained with Schiff's reagent for two hours, the cells were then bleached three times, for two minutes each, in 5% sulfurous acid. After a wash for ten minutes in distilled water the slides were dehydrated through the same series of ethanol washes, dried and mounted.

Data Recording

All cell images were recorded on a Zeiss SMP I scanning microphotometer, operated on line to a PDP 12 computer. The measurements were taken at a wavelength of 540 nm, and a scanning spot size of 0.5 x 0.5 μ was used throughout. Immediately after recording a cell image, its digitized image was displayed as a point array on a CRT, and points within the scanfield which were unrelated to the desired lymphocyte image were edited out by means of a joystick controlled eraser cursor (51). The edited cell images were then transferred to 7 channel tape, and analyzed on the PDP 11/45 computer at the Pharmacy--Microbiology Building, using the TICAS - 11/45 monitor program. All recorded data were stored on magnetic tape to be permanently available in the lymphocyte cell image data bank. The amount of Feulgen chromohor is

measured as the sum over all of the digitized optical densities recorded in the cell image. This sum is often also referred to as "total optical density," or total O.D. Only values exceeding a noise threshold in O.D., typically set at 0.01 O.D. are included. To be able to store all digitized images in the most efficient form in the computer, the actual optical density values, which range from 0.00 to 2.56, are all multiplied by 100, so that they can be represented by integer numbers.

Statistical Evaluation

A total of 10,400 cell images were analyzed in this study. Cell "samples" consisted of sets of 63 cells each, the reason for this odd number is that each LINC tape, used to record the data on the PDP 12 computer accepts that many cells in its index directory. In establishing the significance of various experimental results appropriate designs were chosen. In several cases it was of interest to estimate the significance of an interaction term, and a factorial arrangement of treatments was then chosen. Since the nature of the data was such that a mixed model applies often (52), the interaction mean square was then used as denominator in the F-test for establishment of significance levels, since it properly represents the sampling error, rather than the 63 variable values obtained from the individual cells, which error term is more like a subsampling error, and estimates the precision of the microphotometric determination. In other cases a nested design was chosen (53).

The unsupervised learning algorithm PINDEX (45), a potential function method, is tested by Beale's test statistic (54) for

multivariate significance of a derived partitioning. This test statistic is asymptotically distributed as an F-statistic, and is computed as follows:

$$F(ci,cj) = \frac{R_{ci} - R_{cj}}{R_{cj}} \quad \frac{n - cj}{n - ci} \quad \frac{ci}{cj}^{2/p} - 1$$

In this, R_{ci} and R_{cj} represent the minimum squared error criterion for the partitioning of a data set into ci, or cj subsets respectively. n is the sample size, and p is the dimensionality.

F (ci,cj) is tested as an F-ratio with p(ci-cj), and p(n-ci) degrees of freedom.

Practically all data sets were processed by the PINDEX algorithm, but in only very few instances did significant partitionings result. The significance level for partitioning of a multivariate data set should be chosen considerably higher than is customary in univariate hypothesis testing.

The total amount of Feulgen stain in a cell nucleus is being used as a measure of the amount of DNA present. To establish estimates for the variations expected in this important parameter when different staining batches, different animals, and different cells are involved, an analysis of variance was completed. From each of the three major experiments of this study the data collected on day one, i.e., from the untreated control material were combined. These cells had been stained by three different staining batches, and a total of 15 animals, 5 per batch were available. The design chosen was a nested, mixed model design. The three staining batches lead to 3 - 1 = 2 degrees of freedom

for the among group mean square. For the 5 animals per batch, and the loss of one degree of freedom per batch, this leaves 15 - 3 = 12 degrees of freedom for the subgroup's mean square. From each animal 63 cells were measured, for a total of 15 x 63 = 945 cell images. This means that there are 930 degrees of freedom left for the within subgroup error term. Table 1 shows the analysis of variance table.

The table shows that for this variable, the staining procedure leads to variations which are significant when compared to the animal to animal variations, and also that the cell to cell variation, or the subsampling error, is much smaller than the animal to animal variation. In all following analyses of data, only results derived from cells stained with the same staining batch are compared, so that the batch to batch variability does not enter.

Expressing the expected effects as coefficients of variations one arrives at the following estimates for the different sources of variation:

batch to batch	29.2%
animal to animal	33.4%
cell to cell	1.8%

These estimates are based on the respective mean squares obtained from Table 1.

Table 1. Analysis of Variance Table, Total Amount of Feulgen Stain Per Cell Nucleus Among Controls

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value		alpha
Total ss	944	55,784,450				
Group ss Batch to Batch	2	5,200,030	2,600,015	2 12	0.76	n.s.
Subgroup ss Animal to Animal	12	41,053,680	3,421,140			
Subsubgroup ss Cell to Cell	930	9,530,740	10,248	12 930	333.83	<0.001

EXPERIMENTAL RESULTS

In the first experiment cells were obtained from 6 animals, and collected for a period of eight days in 24 hour intervals. Daily, the animals had been treated by an application of 2,4 DNCB to the neck.

This treatment results in a marked effect on the total amount of Feulgen chromophor developing in the nuclei of the peripheral blood lymphocytes. Within 24 to 48 hours the mean value decreased from 5130 AU to 3250 AU (AU = arbitrary units). Within individual animals the decrease may reach much lower values, but there are slight differences in the time of onset among the different animals. This initial decrease is followed by a return to almost normal values, and by another decline. Fig. 1 shows the effect.

An analysis of variance was carried out to establish the significance of these changes. A two level factorial mixed model design was chosen. Table 2 represents the results.

Both the animal to animal, and the time and duration of treatment show significant effects, and the interaction term is significant. The significance of this latter term reflects the variation in the onset of the decreasing amount of Feulgen chromophor found in different animals. The confidence limits shown in Fig. 1 are based on the mean square of the interaction term, and a sample size of 6 animals, with 63 cells each.

While there thus exist considerable differences in the response among different animals, as shown by the significance of the between

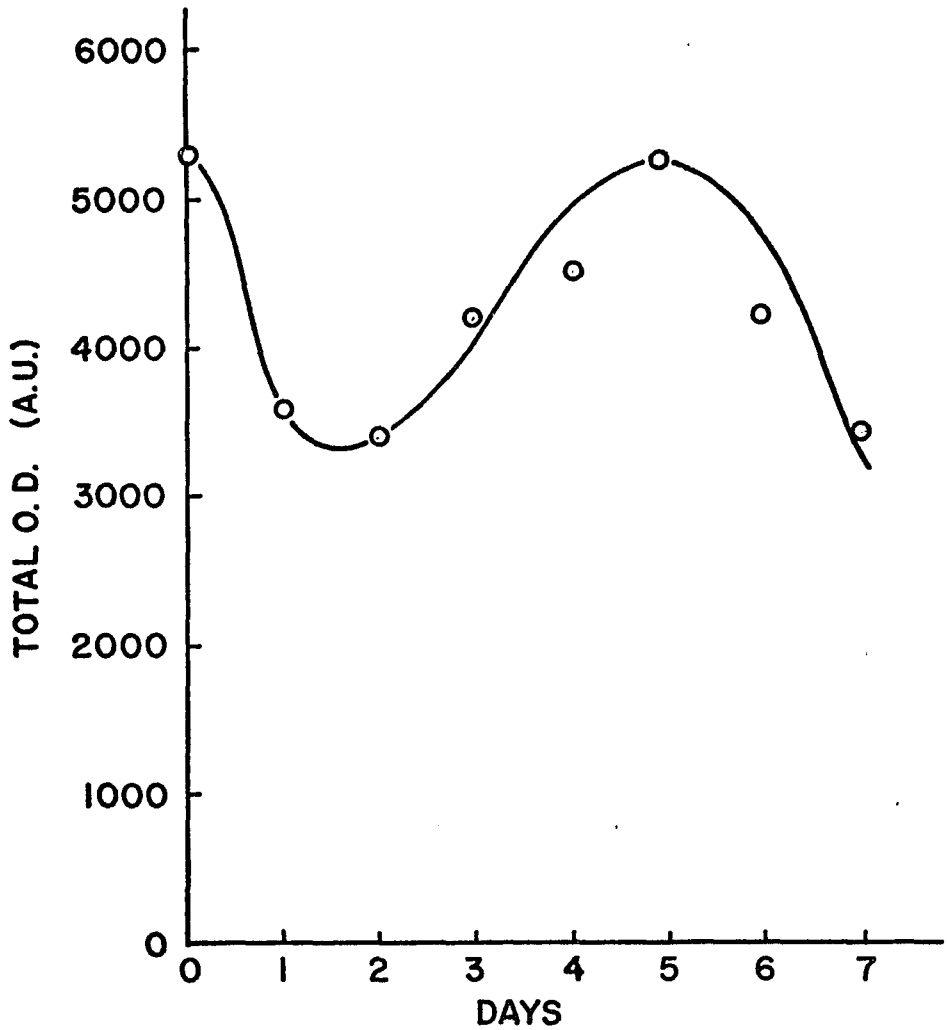

Fig. 1. Total Optical Density of Lymphocytes from Six Guinea Pigs Treated with DNCB

Table 2. Analysis of Variance Table, Effects of DNCB Treatment as a Function of Time, on Total Optical Densities

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	alpha
Total ss	3,023	7,162,765,950			
All Treatments ss	47	5,717,956,400			
A time ss	7	2,083,589,197	297,655,596	$^{7}_{35}$ 4.710	<0.005
B animal ss	5	1,422,910,297	284,582,059	$^{5}_{35}$ 4.504	<0.005
AB time x animal ss	35	2,211,456,906	63,184,483		
Error term ss	2,976	1,444,809,550	485,487	$^{35}_{2976}$ 130.14	<0.001

animal, and the interaction term mean square in Table 2, these differences appear to be of a quantitative nature, rather than in quality. Figs. 2, 3, and 4 show that with the exception of a single animal, a qualitatively similar response occurs.

Also shown are two curves obtained from a repeat experiment. These cells were stained in a different staining batch, and even though the mean value for the control samples fell into the confidence region of the controls in the experiment described above, the data from the repeat experiment were not included in the analysis of variance.

The relative area of the nucleus exhibits a very similar trend, with a weak minimum after three days of treatment followed by a return to the initial values and again by a decrease. This is shown in Fig. 5.

The granularity of the chromatin material in the nucleus follows a very similar pattern of change as well. This is shown, by way of example for data sets recorded on animal G3. In this animal, the minimum of Feulgen staining is reached at day three, and the histogram of optical density values in the cell image clearly exhibits the most pronounced shift to lower values on day three. It then returns to the contour seen in the control sample, to undergo another shift towards low O.D. values at day seven (Fig. 6).

In animal G5 the response set in somewhat earlier, on day one. To explore whether the observed changes affect all lymphocytes, or whether possibly different fractions exist, the distribution of total optical density values were plotted, and are shown for animal G5 in Fig. 7.

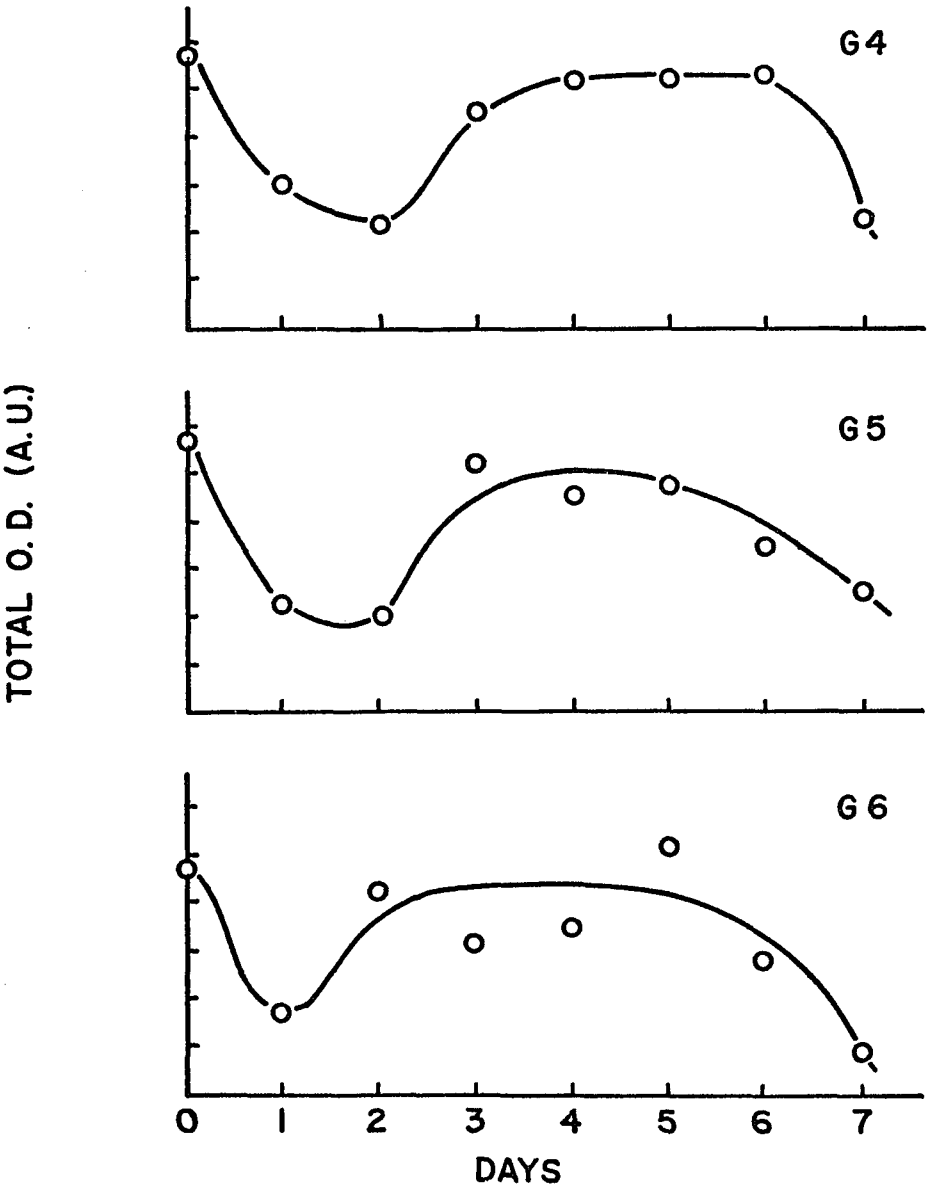

Fig. 2. Total Optical Density of Lymphocytes from Guinea Pigs G4, G5, and G6: Treatment with DNCB

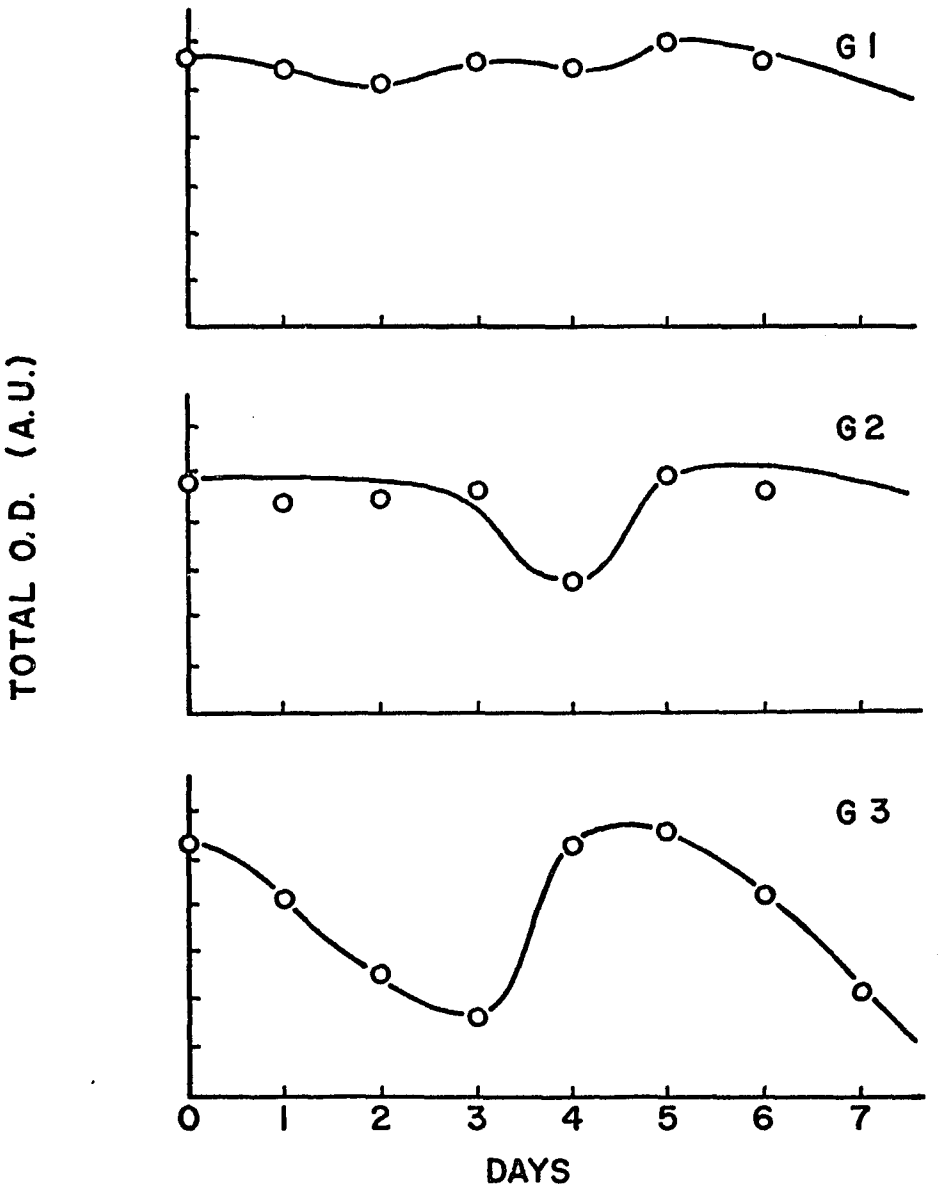

Fig. 3. Total Optical Density of Lymphocytes from Guinea Pigs G1, G2, and G3: Treatment with DNCB

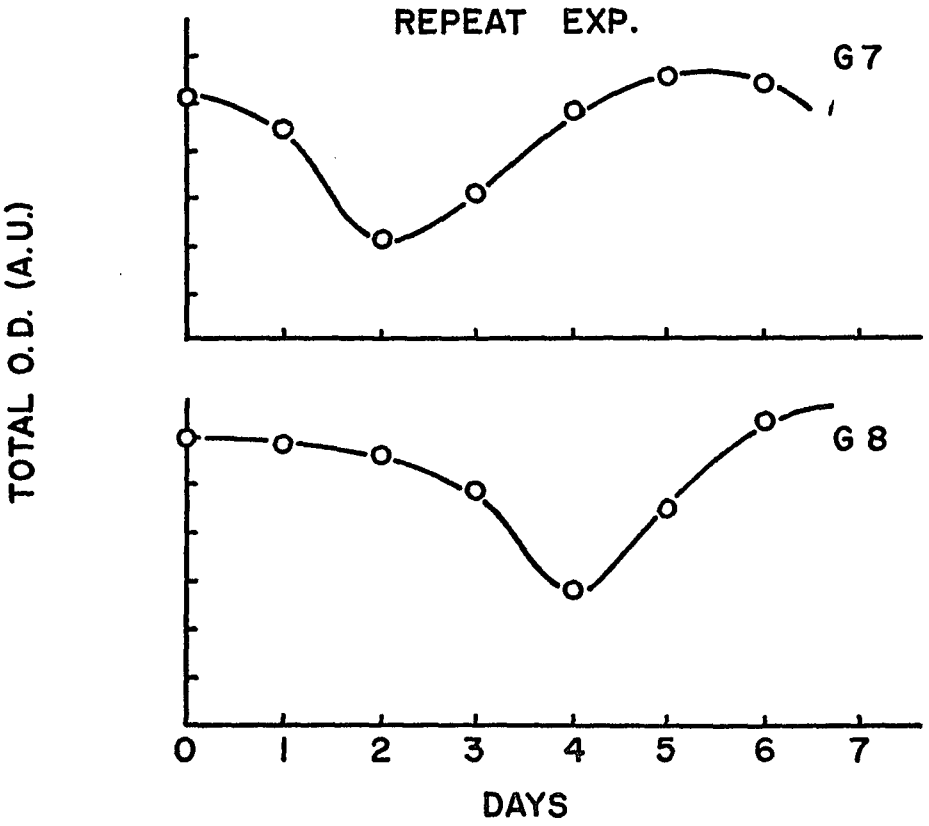

Fig. 4. Total Optical Density of Lymphocytes from Guinea Pigs G7 and G8: Treatment with DNCB

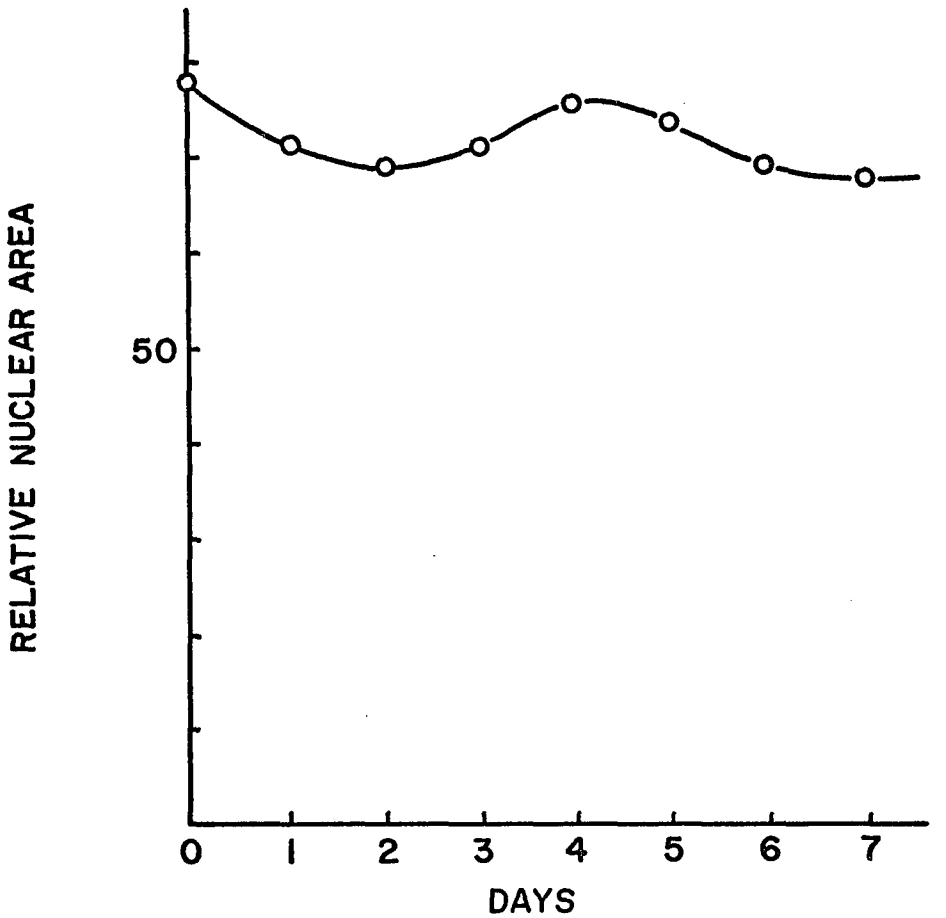

Fig. 5. Relative Nuclear Area of Lymphocytes from Six Guinea Pigs: Treatment with DNCB

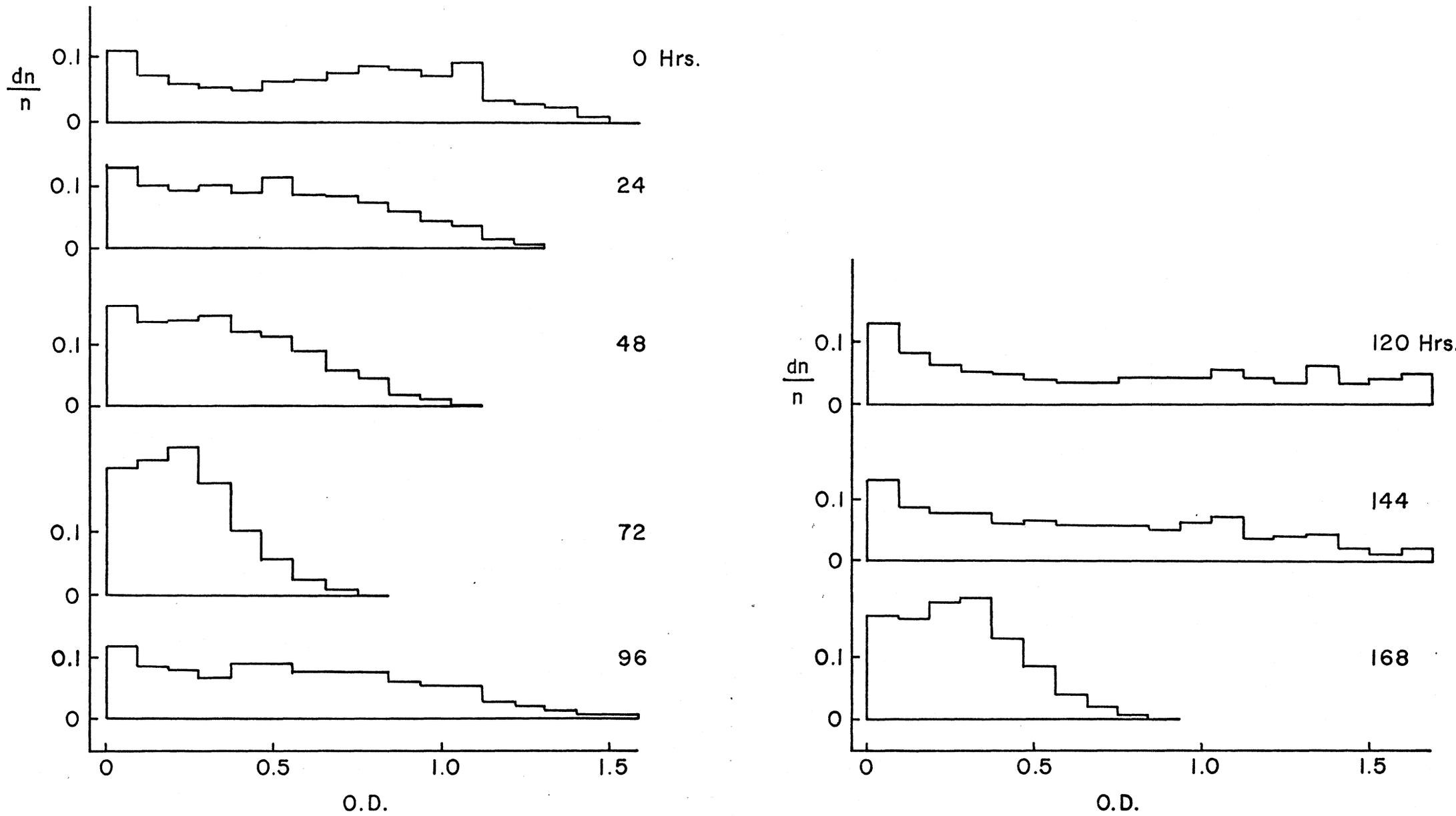

Fig. 6. Histograms of Optical Densities of
Lymphocyte Images from Six Guinea Pigs Treated with DNCB

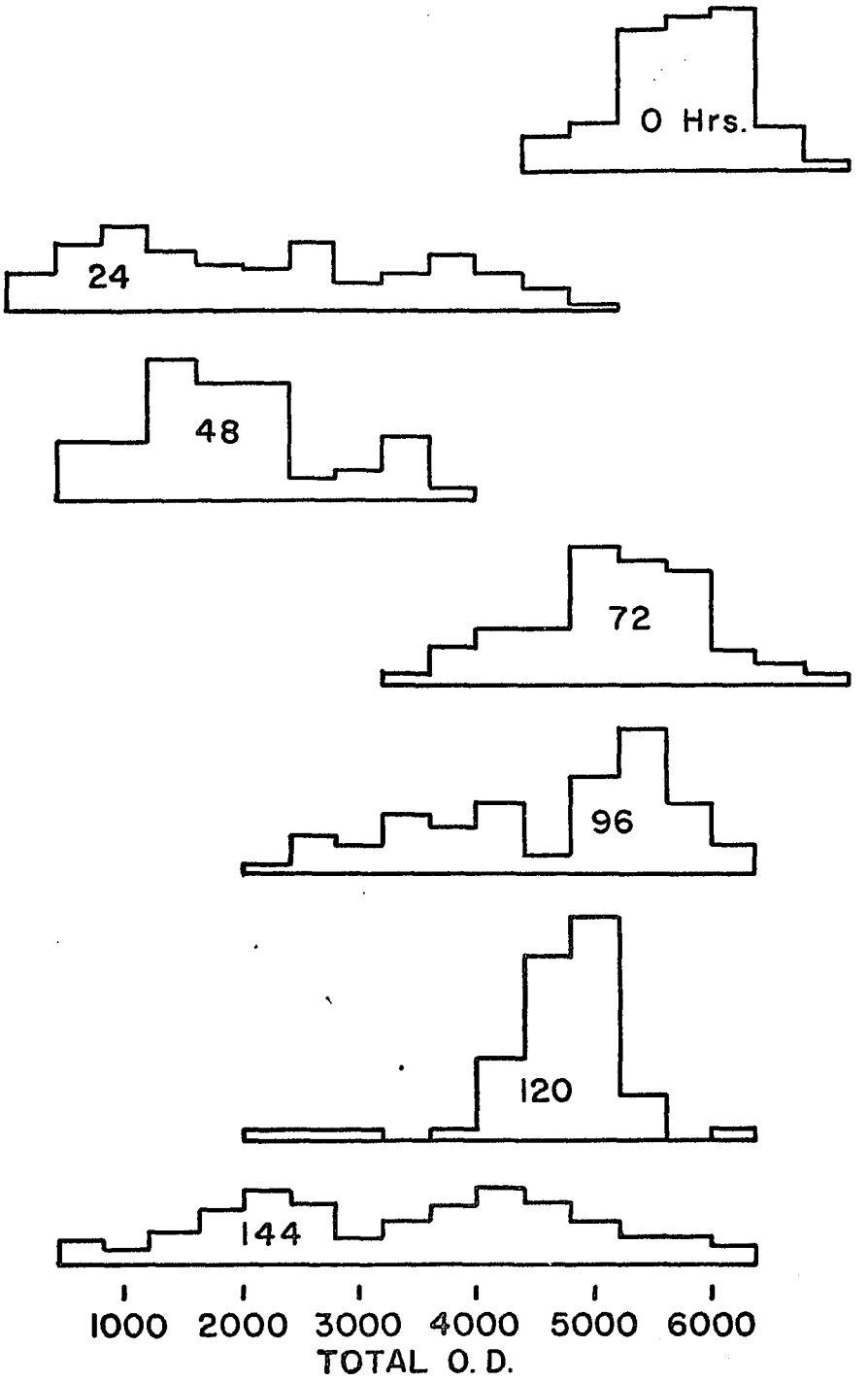

Fig. 7. Distribution of Total Optical Density of Lymphocytes from Guinea Pig G5: Treatment with DNCB

It is evident that every cell in the examined material is affected to some extent, with some cells exhibiting extremely low total O.D. values, and others showing just a slight reduction of the amount of Feulgen staining material. There is no indication that a sizable proportion of cells is not affected at all. There are indications though that in fact two subsets of cells might exist which respond at slightly different rates, or to slightly different extent. This is seen in the apparent bimodality of the distributions seen after one day, and again after six days.

The sample size of 63 cells is too small to permit definite conclusions, for the cells from this one animal alone. However, to examine the possibility of a heterogeneity of response further, the total O.D. distribution found in cells from animal G5 on day four was analyzed further. It appeared to permit a partitioning into two data sets, with a threshold at 4600 A.U. of O.D.

If one submits the histograms of optical density of the entire set of cell images from animal G5, day four to an unsupervised learning algorithm, a partitioning into two subsets is obtained which appeared to classify cell images into two subsets corresponding to the partitioning made visually on the basis of the total O.D. distribution. A 2 x 2 contingency Table 3 shows the results.

Computing the chi^2, with Yates correction for discrete data, renders a chi^2-value of 18.96, which suggests a strong, and highly significant relationship (55) ($alpha < 0.005$) between the automatic

Table 3. Correspondence Between Total Optical Density and Grouping of Histograms by an Unsupervised Learning Algorithm

	Subgroup 1	Subgroup 2
Total O.D. <4600 A.U.	25	3
Total O.D. >4600 A.U.	11	24

grouping derived on the basis of the histogram contour, and the total O.D. of the cells.

The partitioning derived by the unsupervised learning algorithm, using a six dimensional clustering space, could not be shown to produce two subsets which pass a multivariate significance test for difference: the value of Beale's test statistic is 1.711, and the critical F-ratio for 6, and 366 d.f. respectively is 1.77 for alpha = 0.10. Fig. 8 shows the histograms of the two subsets formed by the unsupervised learning algorithm.

Inspection of the distribution of total optical densities in animal G5 had suggested that a homogeneous, unimodal distribution prevailed at the time when the control samples were taken, but that possibly during the rapid decrease in total Feulgen stainability observed on day one, and again on day six a bimodal distribution might be found.

To explore this lead further, the total O.D. values for the four animals G3, G4, G5, and G6, which exhibited approximately the same timing of the response to DNCB treatment were pooled, and the distributions of total O.D. values were plotted. They are shown, as a function

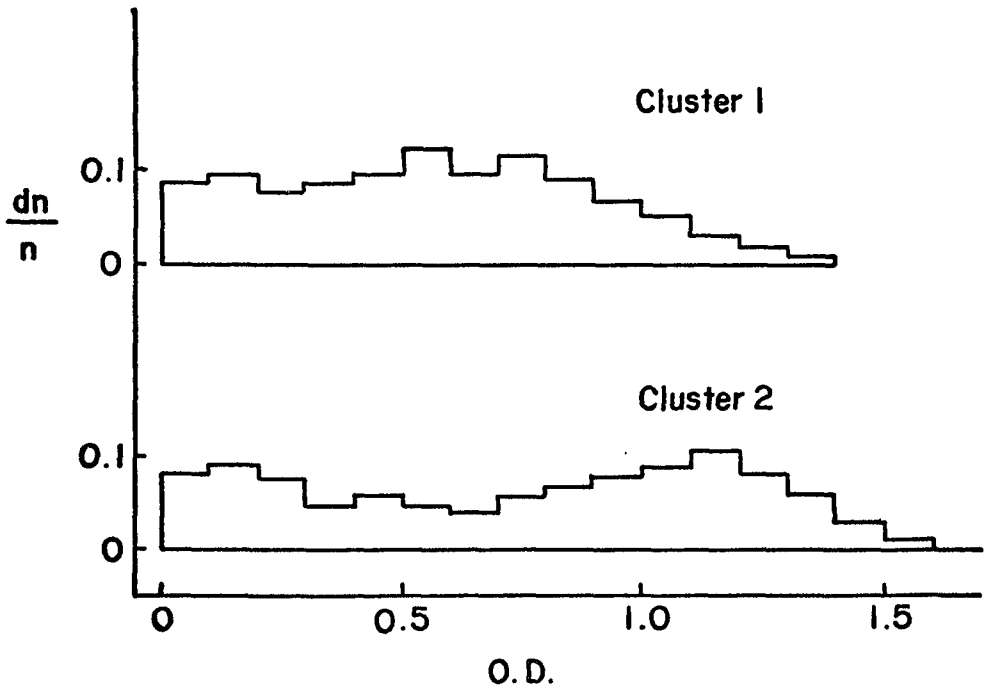

Fig. 8. Histograms of Optical Density of Two Subsets of Lymphocytes from DNCB Treated Guinea Pig G5 Formed by the Unsupervised Learning Algorithm

of time, in Figs. 9-14. The cell files were then subdivided into subfiles of cells with O.D. values falling into the lower modes, or into the high values modes as seen in Fig. 6, and the total O.D. relative nuclear areas, and histograms of these subfile data were computed.

Fig. 15 shows the resulting plot of the computed mean values for the total O.D. values of the detected subsets. The two curves strongly suggest that cell response is indeed heterogeneous, that there are two types of cells with differing sensitivity to the DNCB treatment, but that both show essentially just differential, not qualitatively different response.

It is instructive to examine the histograms of these subsets, as a function of time after treatment. They are shown in Fig. 16 and 17.

The second experiment of this study is similar to the design of the first, it differs in that the animals, in addition to the DNCB treatment, received small doses of the antineoplastic agent 5 FU.

The effect of this on the DNCB induced changes in total O.D. and is shown in Fig. 18.

The very pronounced decrease in Feulgen stain seen in Fig. 1 fails to materialize, and instead, a slight rise by approximately 10% can be observed.

The estimates for the animal to animal mean square presented above suggested that the small increase could possibly not be secured against the biologic variability inherent in the guinea pig response. The observed values for the total amounts of Feulgen stain were

Range	Total	Histogram Plot
100 0000	(1)	I*
100 0000	(5)	I*****
300 0000	(10)	I**********
500 0000	(7)	I*******
700 0000	(10)	I**********
900 0000	(13)	I*************
1100 0000	(8)	I********
1300 0000	(11)	I**********
1500 0000	(11)	I**********
1700 0000	(10)	I**********
1900 0000	(13)	I*************
2100 0000	(6)	I******
2300 0000	(6)	I******
2500 0000	(6)	I******
2700 0000	(7)	I*******
2900 0000	(12)	I**********
3100 0000	(10)	I**********
3300 0000	(6)	I******
3500 0000	(12)	I**********
3700 0000	(15)	I***************
3900 0000	(20)	I********************
4100 0000	(19)	I*******************
4300 0000	(15)	I***************
4500 0000	(9)	I*********
4700 0000	(5)	I*****
4900 0000	(3)	I***
5100 0000	(2)	I**

Fig. 9. Optical Density
Distribution of Lymphocytes from
DNCB Treated Guinea Pigs G3, G4, G5, and G6 on Day One

Range	Total	Histogram Plot
300 0000	(0)	I
500 0000	(5)	I*****
700 0000	(3)	I***
900 0000	(4)	I****
1100 0000	(10)	I**********
1300 0000	(20)	I********************
1500 0000	(12)	I************
1700 0000	(16)	I****************
1900 0000	(18)	I******************
2100 0000	(21)	I**********************
2300 0000	(26)	I**************************
2500 0000	(12)	I************
2700 0000	(15)	I***************
2900 0000	(7)	I*******
3100 0000	(10)	I**********
3300 0000	(16)	I****************
3500 0000	(6)	I******
3700 0000	(10)	I**********
3900 0000	(4)	I****
4100 0000	(5)	I*****
4300 0000	(9)	I*********
4500 0000	(5)	I*****
4700 0000	(7)	I*******
4900 0000	(1)	I*
5100 0000	(3)	I***
5300 0000	(3)	I***
5500 0000	(1)	I*
5700 0000	(1)	I*
5900 0000	(2)	I**

Fig. 10. Optical Density
Distribution of Lymphocytes from
DNCB Treated Guinea Pigs G3, G4, G5, and G6 on Day Two

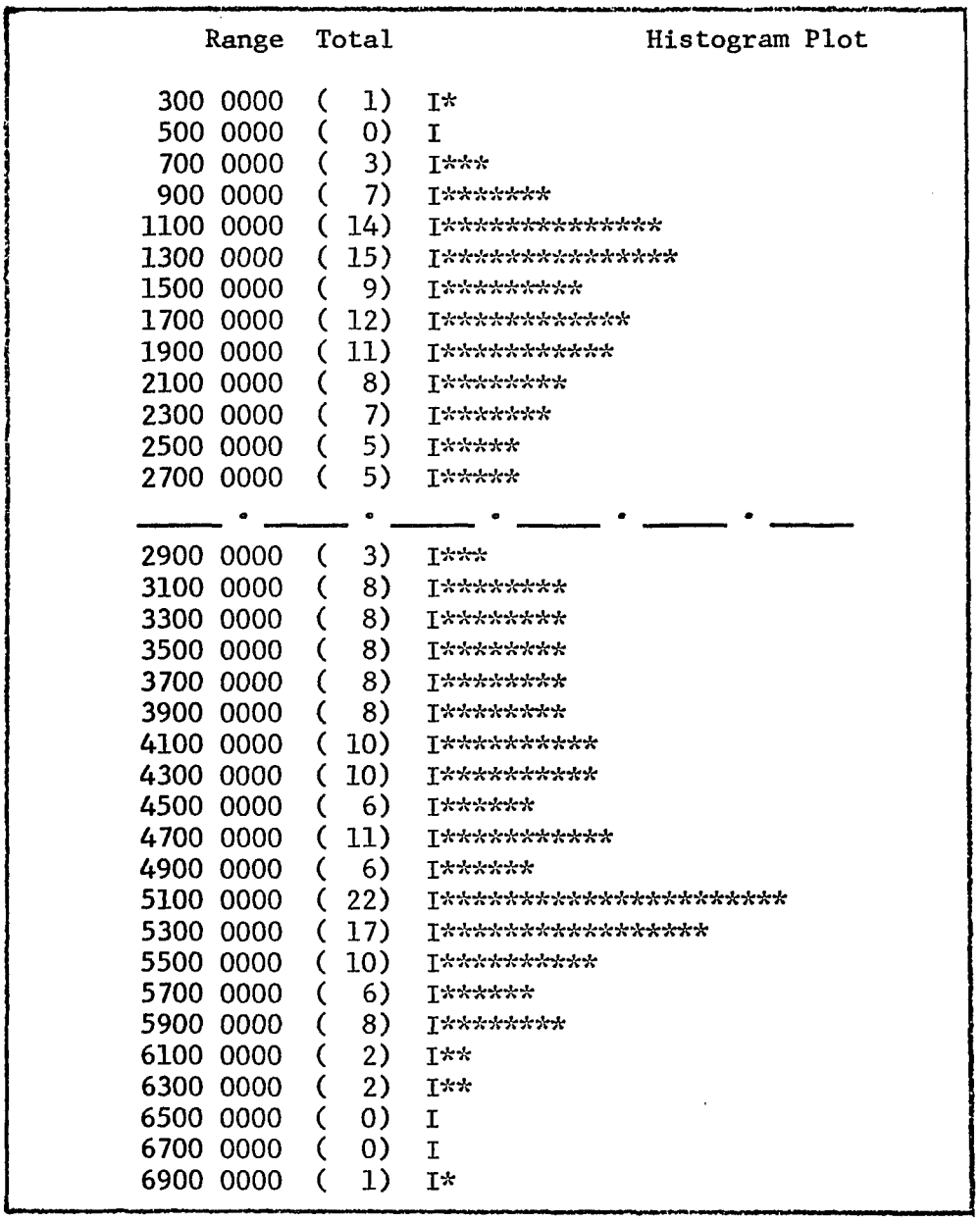

Fig. 11. Optical Density
Distribution of Lymphocytes from
DNCB Treated guinea pigs G3, G4, G5, and G6 on Day Three

Range	Total	Histogram Plot
1700 0000	(1)	I*
1900 0000	(1)	I*
2100 0000	(2)	I**
2300 0000	(3)	I***
2500 0000	(7)	I*******
2700 0000	(3)	I***
2900 0000	(10)	I**********
3100 0000	(8)	I********
3300 0000	(17)	I*****************
3500 0000	(11)	I**********
3700 0000	(11)	I**********
3900 0000	(10)	I**********
4100 0000	(6)	I******
4300 0000	(3)	I***
4500 0000	(7)	I*******
4700 0000	(24)	I************************
4900 0000	(35)	I**********************************
5100 0000	(32)	I********************************
5300 0000	(24)	I************************
5500 0000	(17)	I*****************
5700 0000	(12)	I**********
5900 0000	(6)	I******
6100 0000	(2)	I**

Fig. 12. Optical Density Distribution of Lymphocytes from DNCB Treated Guinea Pigs G3, G4, G5, and G6 on Day Four

Range	Total	Histogram Plot
2100 0000	(0)	I
2300 0000	(2)	I**
2500 0000	(0)	I
2700 0000	(1)	I*
2900 0000	(1)	I*
3100 0000	(1)	I*
3300 0000	(2)	I**
3500 0000	(3)	I***
3700 0000	(1)	I*
3900 0000	(2)	I**
4100 0000	(7)	I*******
4300 0000	(13)	I*************
4500 0000	(16)	I****************
4700 0000	(41)	I**
4900 0000	(45)	I**
5100 0000	(32)	I**********************************
5300 0000	(29)	I*****************************
5500 0000	(25)	I************************
5700 0000	(12)	I************
5900 0000	(7)	I*******
6100 0000	(4)	I****
6300 0000	(5)	I*****
6500 0000	(0)	I
6700 0000	(0)	I
6900 0000	(0)	I
7100 0000	(0)	I
7300 0000	(0)	I
7500 0000	(0)	I
7700 0000	(1)	I*
7900 0000	(1)	I*
8100 0000	(0)	I
8300 0000	(0)	I
8500 0000	(0)	I
8700 0000	(0)	I
8900 0000	(0)	I
9100 0000	(0)	I
9300 0000	(0)	I
9500 0000	(0)	I
9700 0000	(0)	I
9900 0000	(0)	I
10100 0000	(0)	I
10300 0000	(1)	I*

Fig. 13. Optical Density
Distribtuion of Lymphocytes from
DNCB Treated Guinea Pigs G3, G4, G5, and G6 on Day Five

Range	Total	Histogram Plot
500 0000	(1)	I*
700 0000	(1)	I*
900 0000	(2)	I**
1100 0000	(4)	I****
1300 0000	(6)	I******
1500 0000	(10)	I**********
1700 0000	(5)	I*****
1900 0000	(4)	I****
2100 0000	(9)	I*********
2300 0000	(20)	I********************
2500 0000	(7)	I*******
2700 0000	(10)	I**********
2900 0000	(13)	I*************
3100 0000	(6)	I******
3300 0000	(7)	I*******
3500 0000	(6)	I******
3700 0000	(8)	I********
3900 0000	(12)	I************
4100 0000	(5)	I*****
4300 0000	(14)	I**************
4500 0000	(12)	I************
4700 0000	(22)	I**********************
4900 0000	(20)	I********************
5100 0000	(17)	I*****************
5300 0000	(14)	I**************
5500 0000	(10)	I**********
5700 0000	(4)	I****
5900 0000	(0)	I
6100 0000	(2)	I**
6300 0000	(0)	I
6500 0000	(0)	I
6700 0000	(1)	I*

Fig. 14. Optical Density
Distribution of Lymphocytes from
DNCB Treated Guinea Pigs G3, G4, G5, and G6 on Day Six

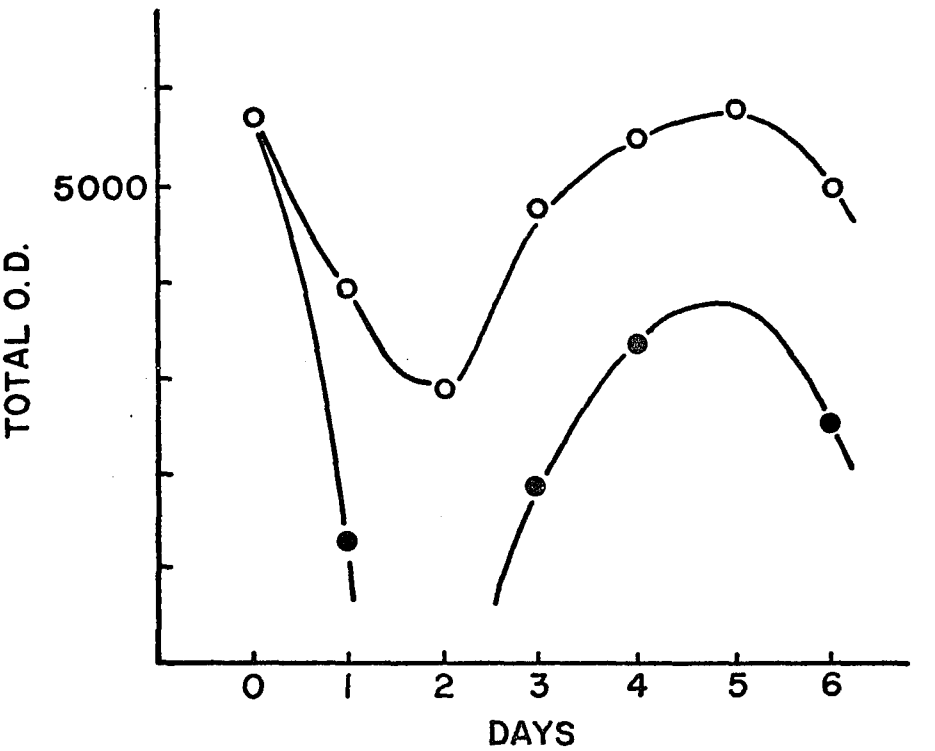

Fig. 15. Total Optical Densities of
Subsets of Lymphocytes from DNCB Treated Guinea Pigs

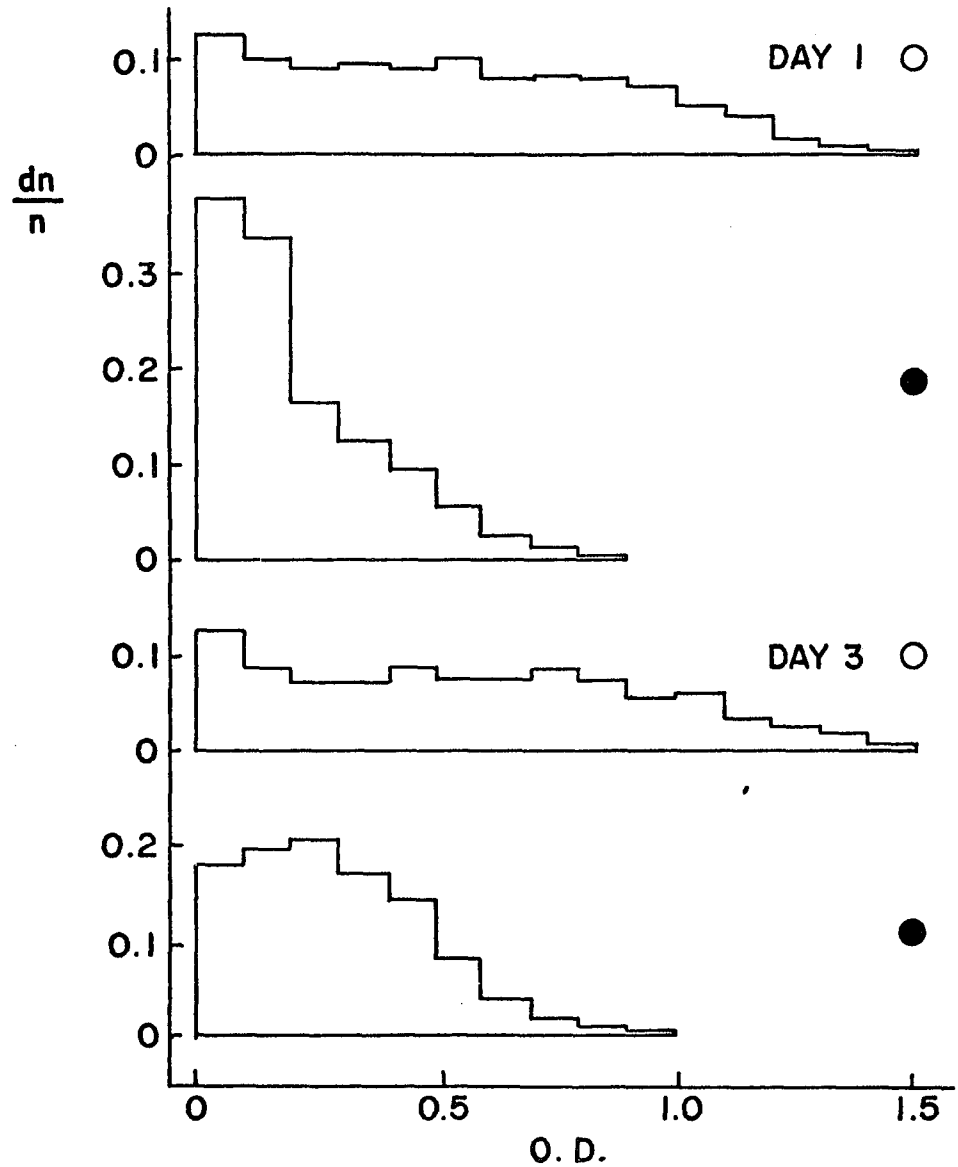

Fig. 16. Histograms of Optical Densities for Subsets of Lymphocytes Found in Guinea Pigs Treated with DNCB: Comparison of Subset with Low Optical Density to Subset with High Optical Density, Day One and Day Three

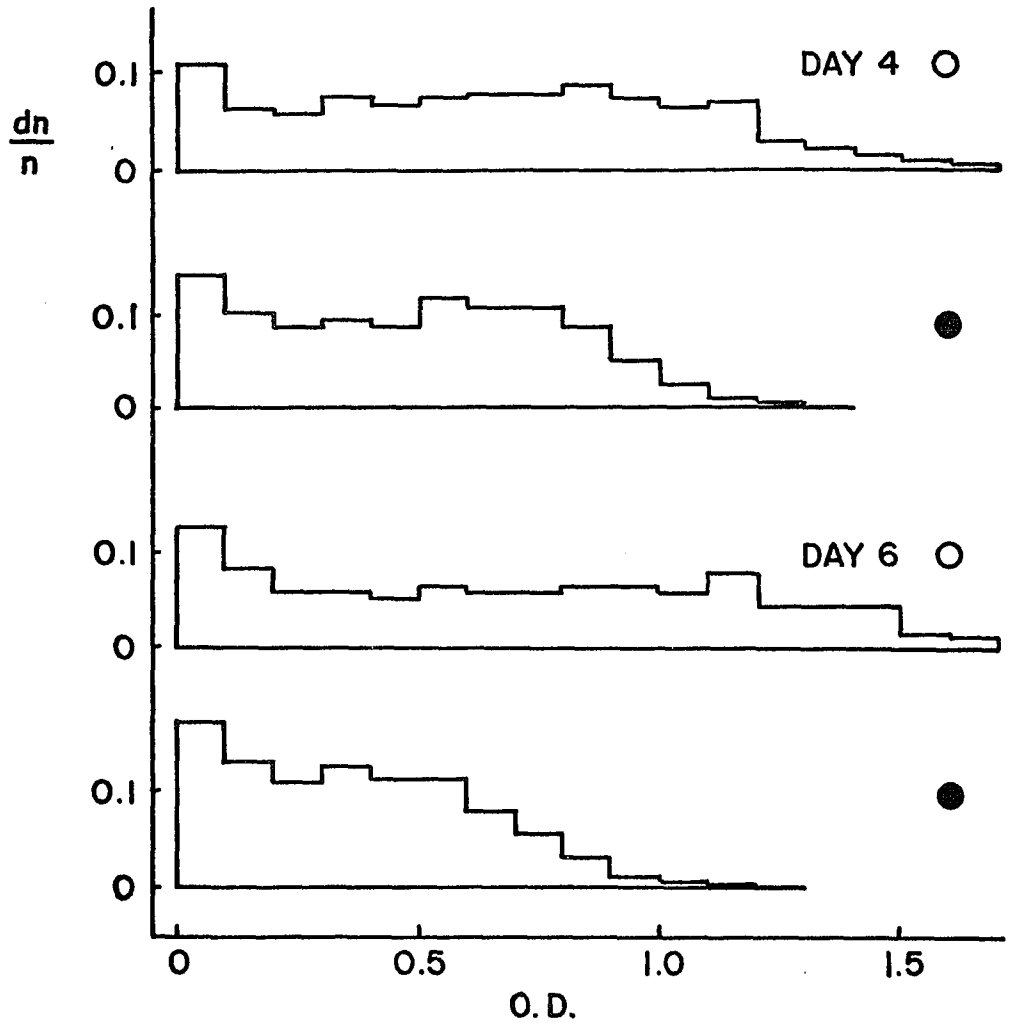

Fig. 17. Histograms of Optical Densities for Subsets of Lymphocytes Found in Guinea Pigs Treated with DNCB: Comparison of Subset with Low Optical Density to Subset with High Optical Density, Day Four and Day Six

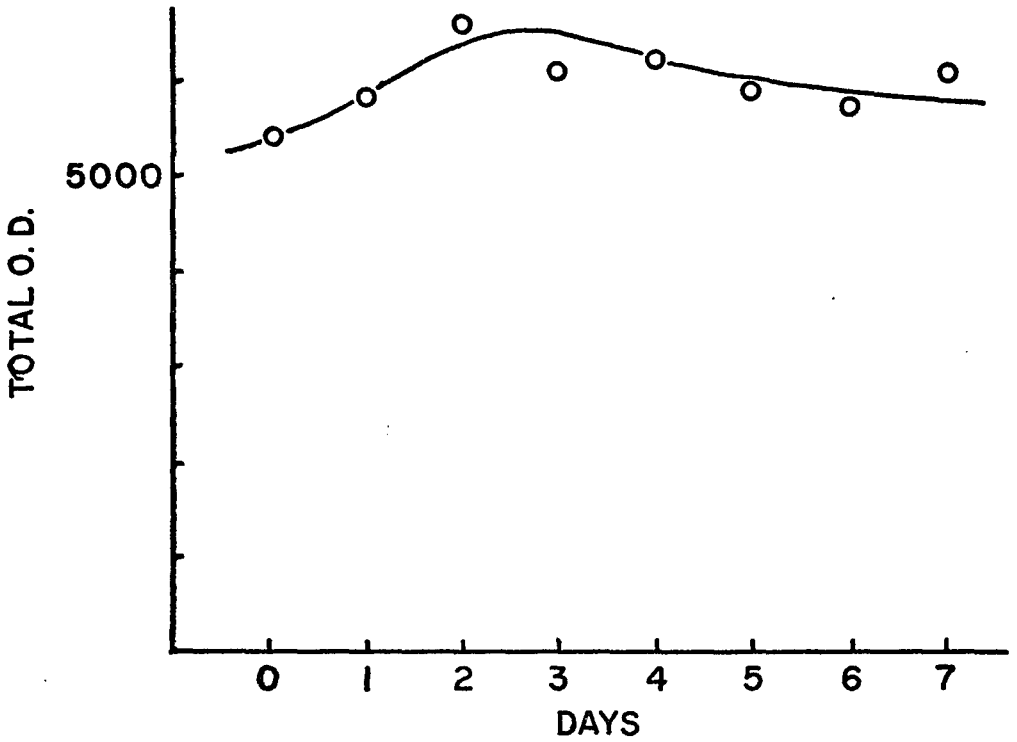

Fig. 18. Total Optical Density Plot of Lymphocytes from Guinea Pigs Treated with DNCB and 5 FU

therefore subjected to an anlysis of variance. The results are presented in Table 4.

The analysis confirms the visual impression. The animal to animal variation marks any significance of the slight increase in staining ability which the graph in Fig. 18 shows to occur between days two and three. The increase could be observed in three out of the four animals in this experiment.

The distribution of O.D. values in individual cells remains quite constant: Fig. 19 shows, by way of example, the histograms of O.D. values for animal G3 for days three and six.

The results from the experiment where only 5FU was given exhibit a particularly noticeable variability. The effect on the total amount of Feulgen stain can be seen in Fig. 20. In all but one of the six animals used in this study a marked decrease in the total amount of Feulgen stain can be observed. An analysis of variance shows that this decrease is significant, again in all but one of the animals, on the sixth day. The interaction term mean square which estimates the sampling error corresponds to a coefficient of variation of 34%, which must be considered as high, and Fig. 20 clearly shows that highly significant differences between the various animals existed. Nevertheless the decrease in total amount of Feulgen stain as a function of time after the initial treatment can be secured as being significant at the 99% level. Table 5 gives the results of the analysis of variance.

The decrease in staining expresses itself also in the histogram of O.D. values in the individual cells. Fig. 21 and 22 show the

Table 4. Analysis of Variance Table, Total Optical Density of Lymphocyte Nuclei from Guinea Pigs Treated with DNCB and 5 FU

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	alpha
Total ss	1763	1,724,723,470			
All Treatments ss	27	1,390,423,470			
A time ss	6	227,420,217	37,903,369	$\frac{6}{18}$ 0.97	n.s.
B animals ss	3	460,647,860	153,549,286	$\frac{3}{18}$ 3.935	<0.05
AB time x animal ss	18	702,355,392	39,019,744	$\frac{18}{1736}$ 202.62	<0.001
Error term ss	1736	334,300,000	192,569		

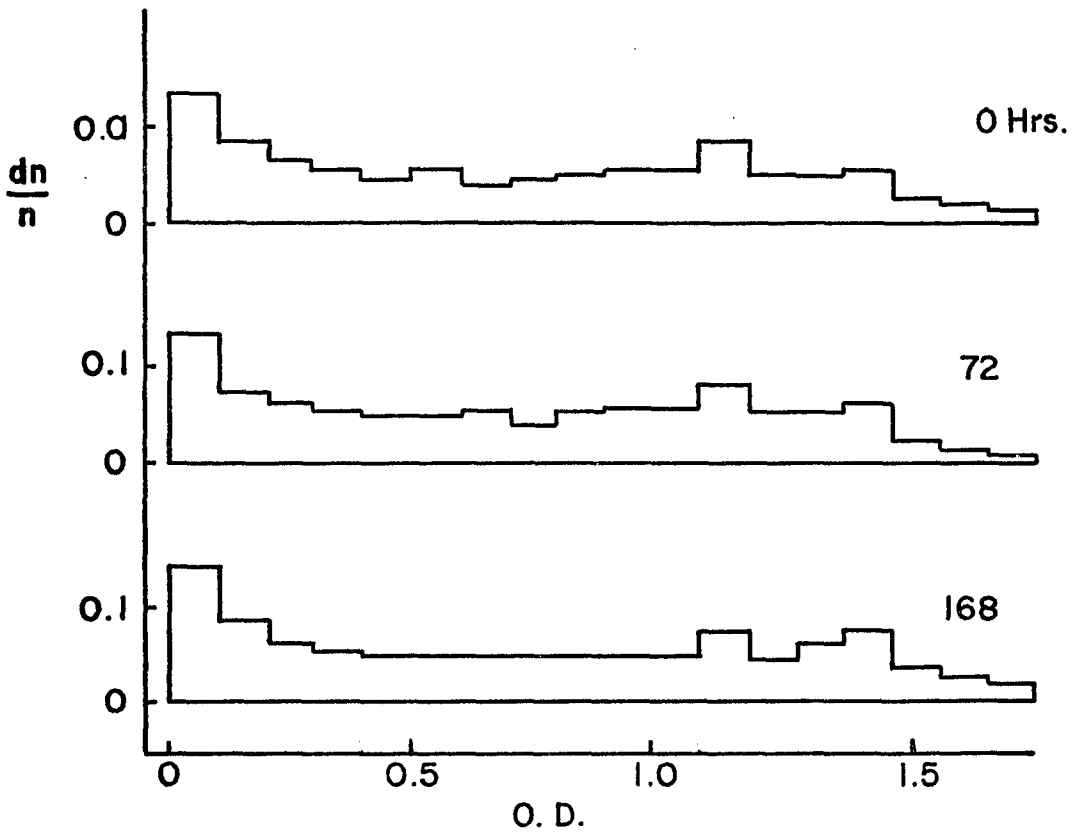

Fig. 19. Optical Density Histograms of Lymphocytes from Guinea Pig G3 Treated with DNCB and 5 FU

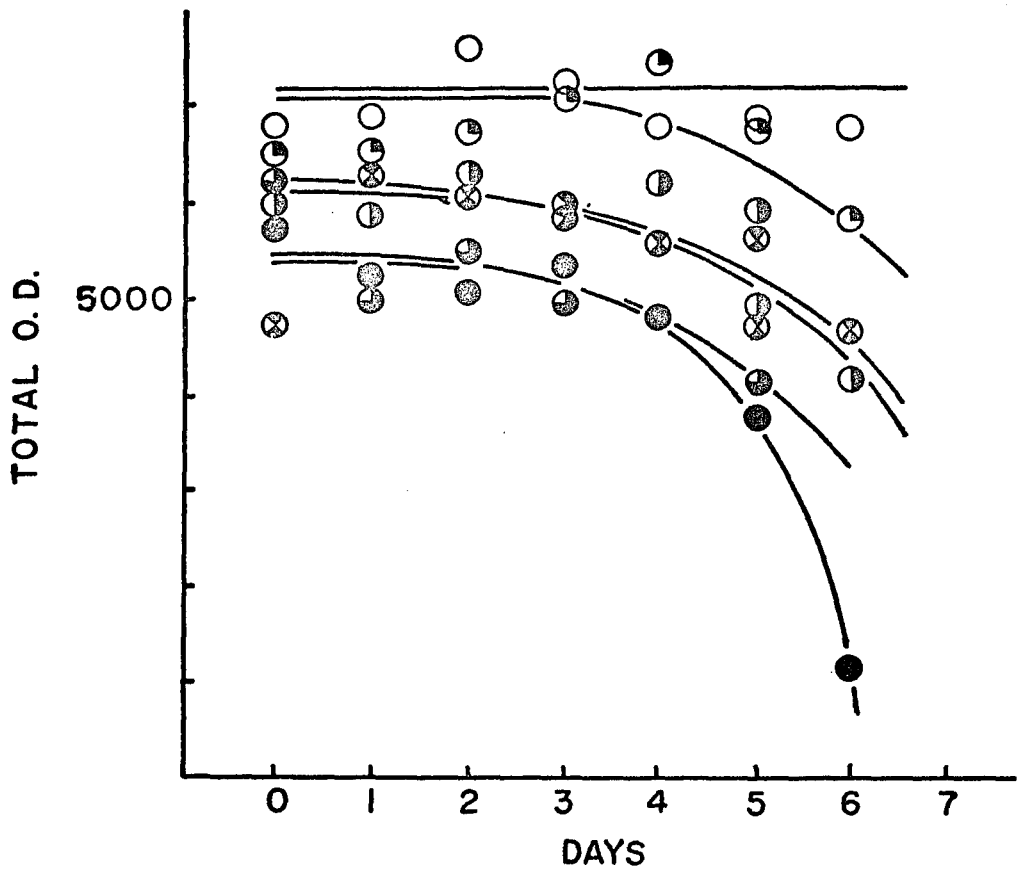

Fig. 20. Total Optical Density of Lymphocytes from Six Guinea Pigs Treated with 5 FU

Table 5. Analysis of Variance Table, Total Optical Density of Lymphocyte Nuclei from Guinea Pigs Treated with 5 FU

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	Alpha
Total ss	2645	7,227,853,270			
All Treatments ss	41	4,450,535,270	108,549,000		
A time ss	6	979,942,056	163,323,000	$\frac{6}{30}$ 3.883	<0.01
B animals ss	5	2,208,832,200	441,766,000	$\frac{5}{30}$ 10.50	<0.001
AB time x animal ss	30	1,261,760,900	42,058,000	$\frac{30}{2604}$ 39.45	<0.001
Error Term ss	2604	2,777,318,000	2,066,558		

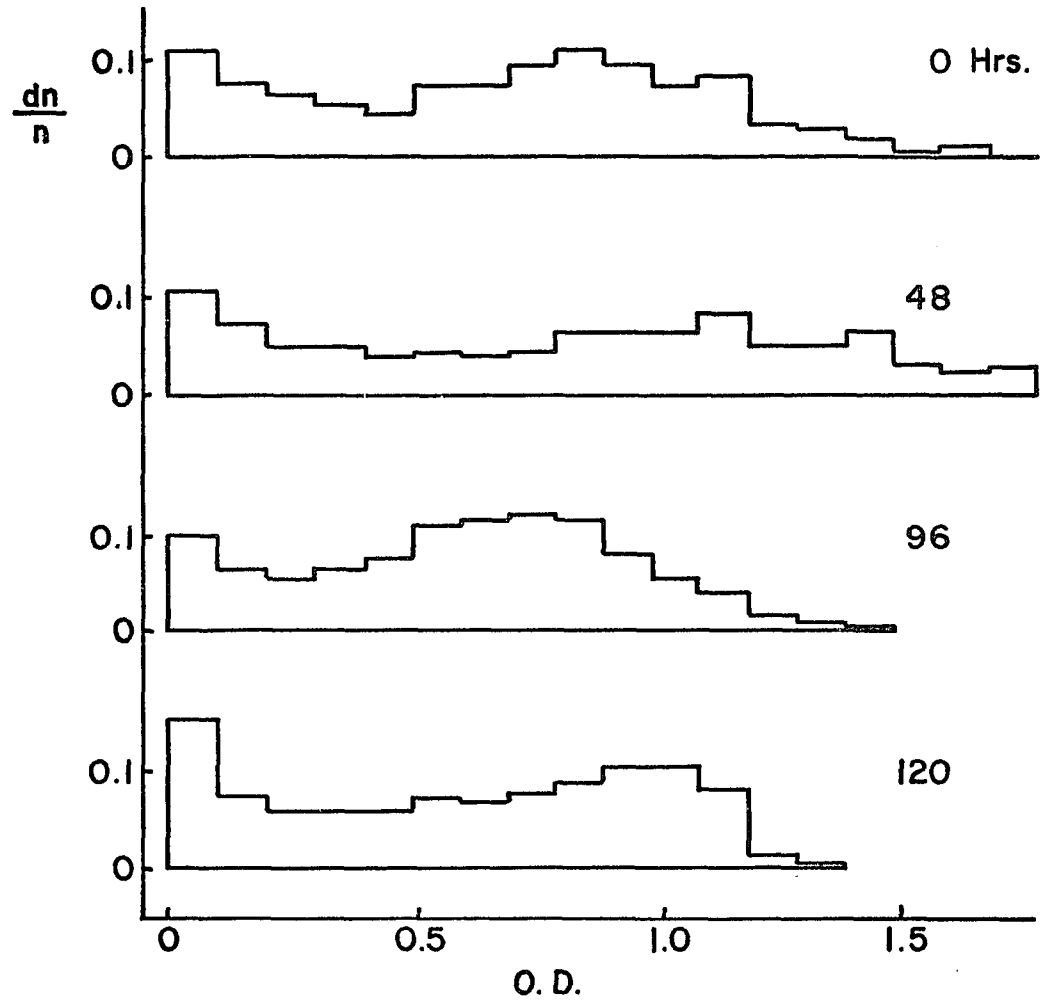

Fig. 21. Optical Density Histograms of Lymphocytes from Guinea Pig G1 Treated with 5 FU

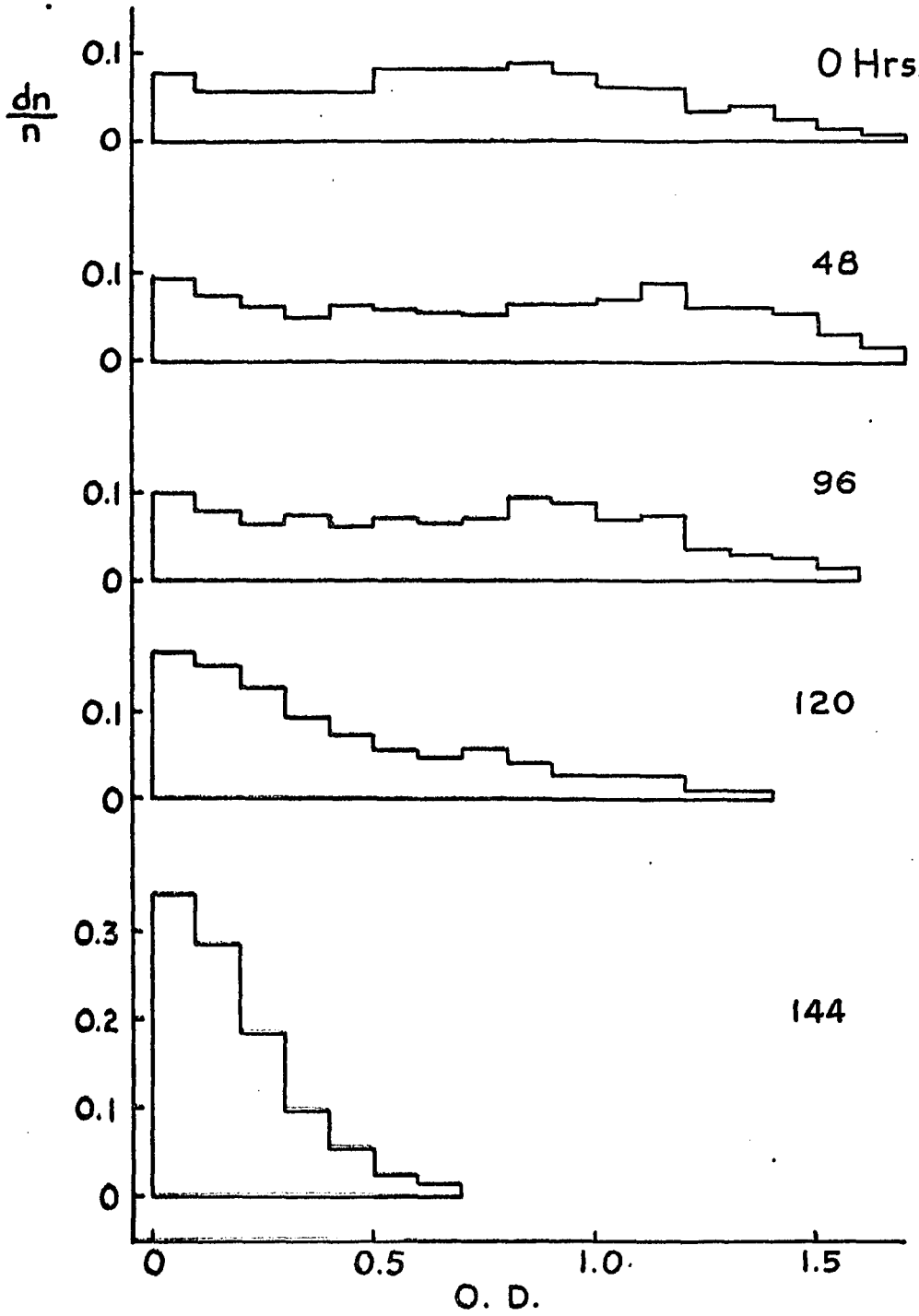

Fig. 22. Optical Density Histogram Profiles of Lymphocytes from Guinea Pig G2 Treated with 5 FU

histograms of O.D. values measured in a set of cells from two animals, as a function of time after initial treatment. Each histogram has been averaged over 63 individual cell histograms.

The final experiment of this study was aimed at a quantitation of changes in peripheral blood lymphocytes after treatment of the animals with cyclophosphamide. A plot of the total optical density values averaged over 63 cells each shows no obvious effect of cyclophosphamide on the development of Feulgen chromophor, at the dose levels employed here. This is seen in Fig. 23. The analysis of variance confirms the conclusion. Table 6 presents the results of the analysis of variance.

Table 6 shows that treatment with cyclophosphamide over a period of six days and at the described dose levels has no significant effect on the total optical density of peripheral blood lymphocytes in the guinea pig. The differences between animals were significantly different from the sampling error, but this did not involve the treated, and the untreated animals. The sampling error is significantly greater than the cell to cell subsampling error. Relating the estimated mean squares back to the total O.D. values, one finds the cell to cell coefficient of variation as 3.4%, the sampling error associated with each set of 63 cells to have a coefficient of variation of 29.3%, and the animal to animal coefficient of variation as 42%.

While treatment with cyclophosphamide under the stated conditions does not lead to a significant change in the amount of Feulgen stain, it appears to have, after approximately two weeks, a noticeable

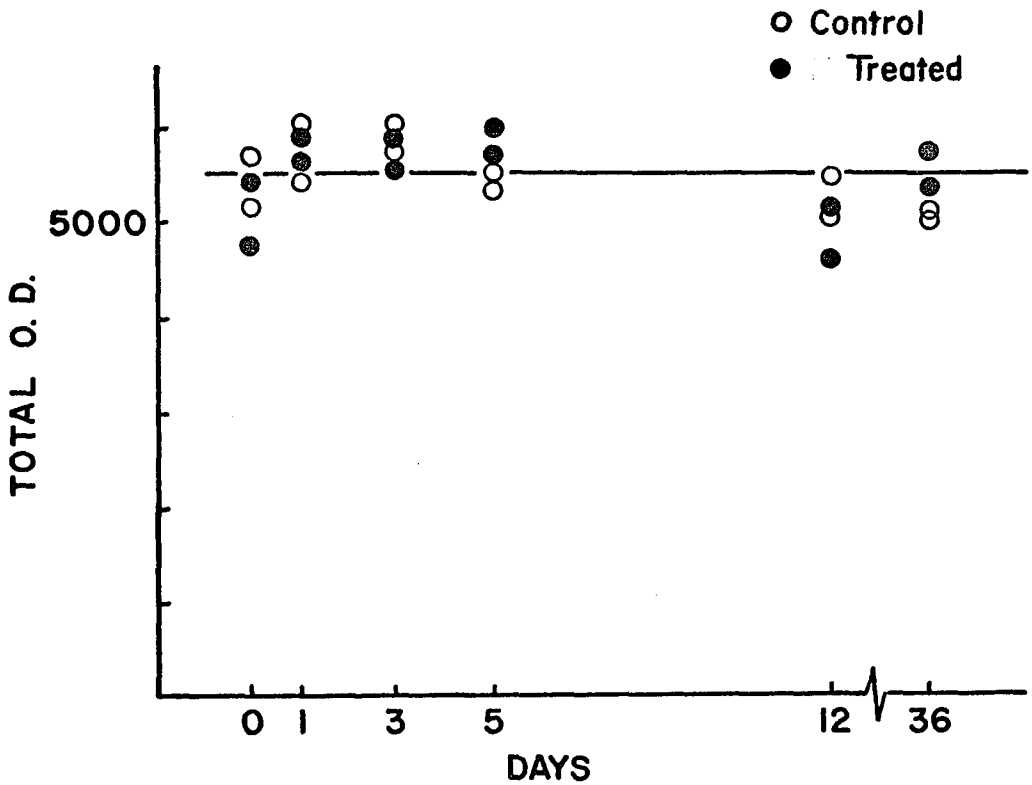

Fig. 23. Total Optical Density Plot of Lymphocytes from Guinea Pigs Treated with Cyclophosphamide

Table 6. Analysis of Variance Table, Total Optical Density of Lymphocyte Nuclei from Guinea Pigs Treated with Cyclophosphamide

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	Alpha
Total ss	1511	1,173,837,450			
All Treatments ss	23	631,517,450			
A time ss	5	80,133,245	16,026,649	$\frac{5}{15}$ 2.84	n.s.
B animals ss	3	466,736,410	155,578,803	$\frac{3}{15}$ 27.56	<0.001
AB time x animal ss	15	84,647,794	5,643,186	$\frac{15}{1488}$ 16.37	<0.001
Error Term ss	1488	542,320,000	344,623		

effect on the chromatin distribution pattern. Fig. 24 shows the histogram of O.D. values of the treated animals as a function of time after initial treatment.

The cell population as a whole appears to undergo this change. When one considers the relative frequency of occurrence of O.D. values in each histogram interval as an image descriptor, one may subject the histogram to a non-parametric statistical variable selection procedure. Such a procedure may be used to determine whether the histogram contours are in fact statistically significantly shifted, and furthermore, which intervals show the greatest differences.

The Kruskal Wallis test (56) is such a procedure. Table 7 shows such a variable selection for the discrimination between the histograms of a control, and histograms from a treated animal, after four weeks of treatment. The program permits the user to specify any pair of suitable variables, and to print a bivariate scattergram. The plots show (Figs. 25, 26) that although the majority of the cells from the animal treated with cyclophosphamide appear to have a changed histogram contour, a small percentage of cells from the treated animal display values as found in the controls.

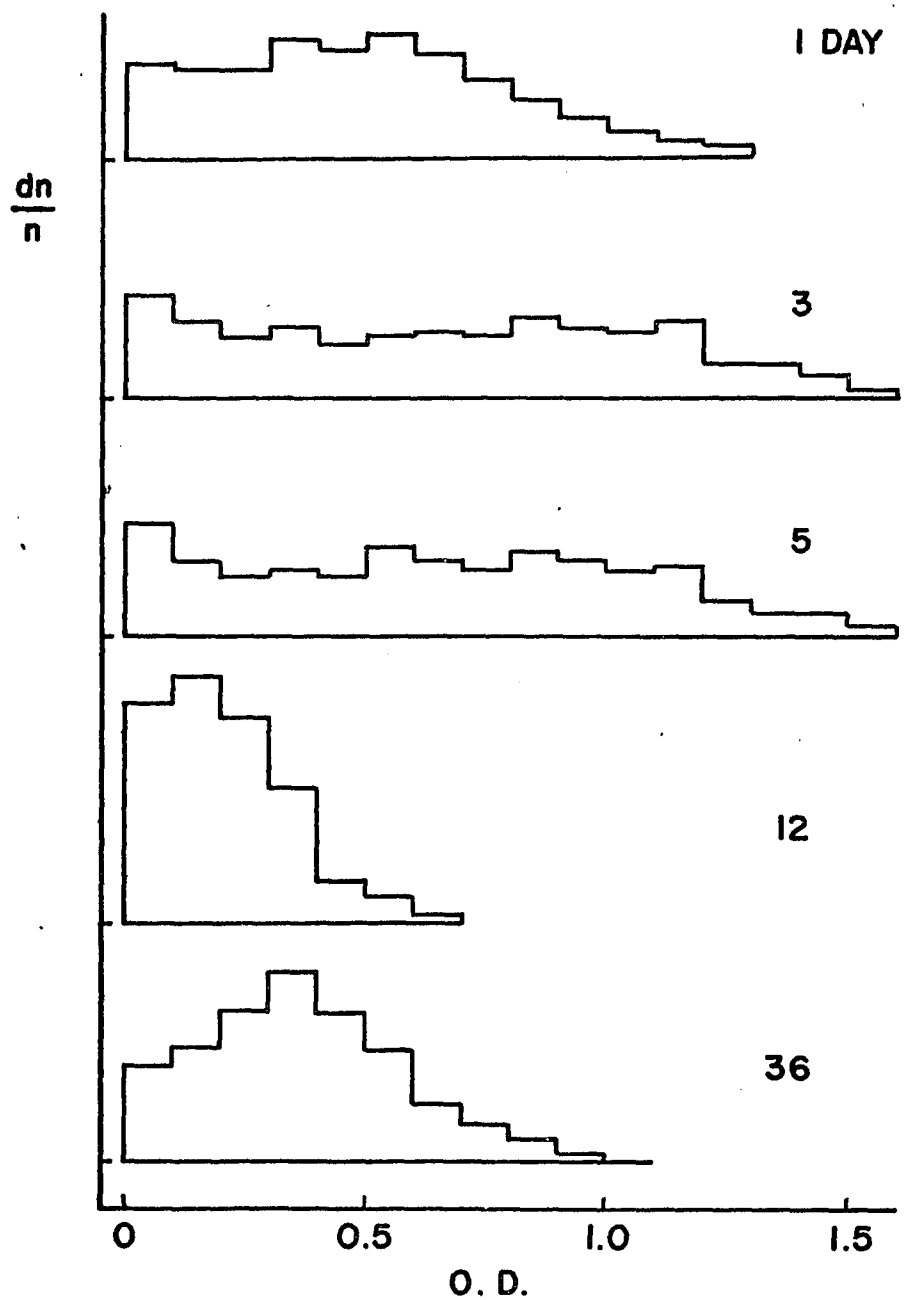

Fig. 24. Optical Density Histograms of Lymphocytes from Guinea Pigs Treated with Cyclophosphamide

Table 7. Kruskal Wallis Test to Select Histogram Intervals with Statistically Significant Differences of Their Relative Frequency of Occurrence Comparison of Data from a Control and an Animal Treated for Two Weeks with Cyclophosphamide

Kruskal Wallis Test Performed on G3517 HST and G3021 HST

Bin Number	Test Statistics H	Cumulative Probability
1	25.26	0.000--0.005
2	40.27	0.000--0.005
3	23.90	0.000--0.005
4	0.1487	0.500--0.750
5	13.48	0.000--0.005
6	43.66	0.000--0.005
7	35.33	0.000--0.005
8	27.79	0.000--0.005
9	19.33	0.000--0.005
10	11.21	0.000--0.005
11	1.741	0.100--0.250
12	0.8842	0.250--0.500
13	0.2548E-01	0.750--0.900
14	0.9476E-01	0.750--0.900
15	0.0000	0.900--1.000
16	0.0000	0.900--1.000
17	0.0000	0.900--1.000
18	0.0000	0.900--1.000

The First 8 Best Bins with Their Corresponding Test Statistics

Bin Number	Test Statistics H	Cumulative Probability
6	43.66	0.000--0.005
2	40.27	0.000--0.005
7	35.33	0.000--0.005
8	27.79	0.000--0.005
1	25.26	0.000--0.005
3	23.90	0.000--0.005
9	19.33	0.000--0.005
5	13.48	0.000--0.005

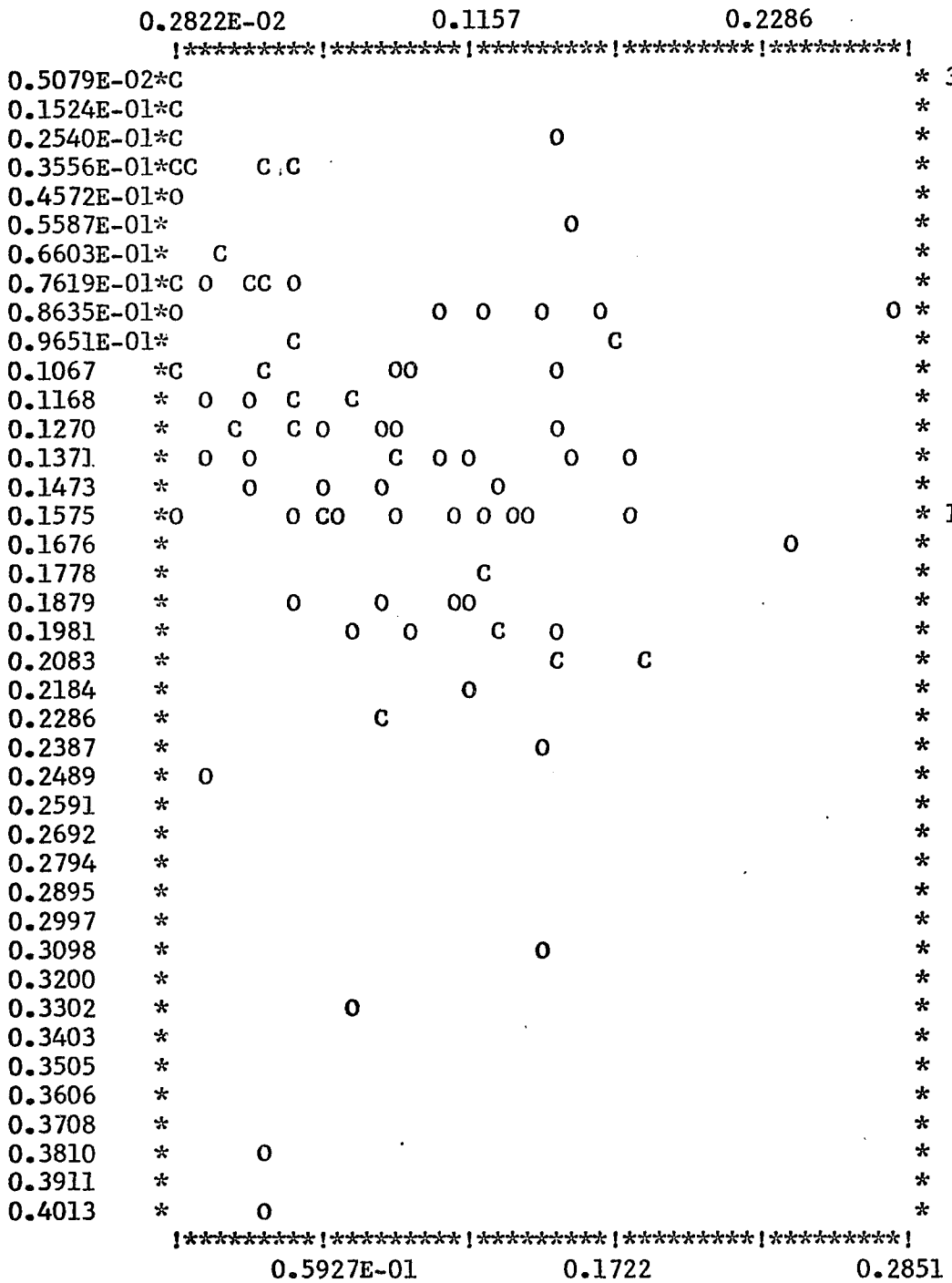

Fig. 25. Bivariate Scattergram
Plot of Relative Frequencies of Occurrence
in Lymphocyte Histograms from Bin Six and Bin
Seven from a Guinea Pig Treated with Cyclophosphamide and Control Animal

O = control; C = two weeks of cyclophosphamide treatment

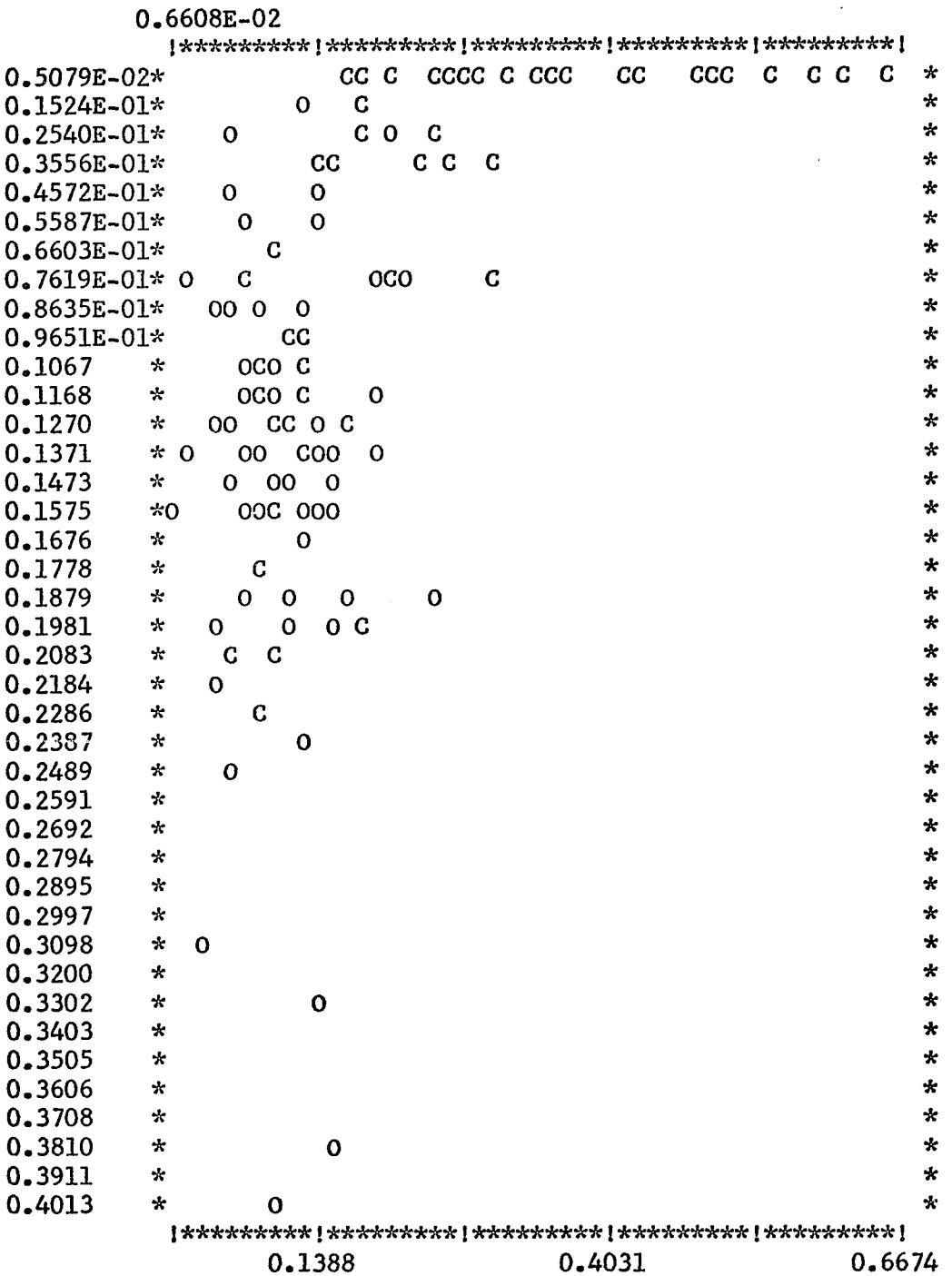

Fig. 26. Bivariate Scattergram
Plot of Relative Frequencies of Occurrence
in Lymphocyte Histograms from Bin Six and Bin Two
from a Guinea Pig Treated with Cyclophosphamide and Control Animal

O = control; C = two weeks of cyclophosphamide treatment

DISCUSSION

The results obtained in this study show that image analytic techniques (57, 58) are suitable to monitor, at the level of individual cells, the response to chemotherapeutic agents. The nature of the findings were surprising in several respects. First, the very pronounced, and general response to an externally applied chemical such as DNCB was not anticipated. Second, the administration of a cytotoxic agent such as 5 FU was expected to produce an early, and clearly registered response. This did not occur. Finally, it had not been expected that the effects of DNCB would be completely inhibited by the simultaneous administration of a small dose of 5 FU. Yet, the results were shown to be reproducible by repeat experiments, and analysis of variance clearly establishes them as significant.

The only results which confirm directly observations made earlier, is the response to treatment with cyclophosphamide. Cyclophosphamide enhances the cell mediated immune response, and had been found to produce in mice, several weeks after treatment, a shift in the average histogram of O.D. values measured in thoracic duct lymphocytes (59). This shift towards lower O.D. values in the histogram of a mixed population of B and T cells in thoracic duct lymphocytes makes the whole histogram resemble the histogram of a purified population of T cells. Earlier work by Bartels, and Jeter had shown that in the peripheral blood of guinea pigs also two sub-populations distinguished by their histogram contour appeared to exist (50) and while in the guinea pig a

subset categorization as "T" or "B" cells has not been established, it has been shown that these subsets resemble in their proportions and histogram contour the T and B cells from chicken, mouse, and in man.

Treatment of guinea pigs with cyclophosphamide over an extended period of time led likewise to changes in histogram contours which suggest an increase in proportion of cells with less extended histograms. On the other hand, similar shifts have occasionally been observed in animals from which blood samples had been taken over a period of time, so that it is not entirely clear at this point that the observed shift is definitely due to the cyclophosphamide treatment.

Two aspects enter into the general interpretation of results. These are first what cytochemical conclusions can be drawn from the observed changes in the amount of Feulgen stain developing in the cells, and second, what are the biologic implications of these changes.

The Feulgen (60) reaction is one of the most widely investigated cytochemical staining procedures. Yet a survey of the literature shows that the exact nature of the processes involved is even today not yet fully understood (61,62). The reaction results in a chromophor which absorbs in the visible range. It has been shown that its total optical density is highly correlated with the total optical densities measured at the peak of DNA absorption at 260 nm in the ultraviolet (63). It has also been shown that even the distribution of O.D. values in the UV, and at the peak of the Feulgen stain absorption are highly correlated. The reaction is considered, under controlled staining conditions, to be stoichiometric for DNA.

Garcia (64) and Gledhill et al. (65) were the first to find apparent deviations from the stoichiometry when cells with highly compacted chromatin were stained, and compared to cells of less maturity, e.g., in spermatocytes. There are however other mechanisms which might affect a systematically denser, or less dense staining. Stoichiometry of the Feulgen stain has traditionally been tested on homogeneous cell populations, and it has been for such homogeneous sets that the optimum hydrolysis time is set and determined. In case of cells of different physiologic function, or of inhomogeneous cell populations in general, say T and B cells, lymphocytes in a mixed population one can not necessarily assume that a given hydrolysis "dose" will lead to comparable effects in all sub-populations. Duijndam published an exacting study into the causes for differing chromophor (66) development in cells with varying chromatin compactness. Noeske (67) found that histones reduced Feulgen values in cells with the most compacted chromatin, and that histin extraction prior to the staining phase of the Feulgen reaction led to uniform, and generally higher Feulgen staining even within cells of such differing maturity as the series as myeloblast, promyelocytes, myelocyte, band and polymorphonuclear cells. It has been postulated by Duijndam that chromatin compactness differences play a role predominantly in the depurination reaction, and not in the staining phase of the Feulgen process, and that histones interfere with the kinetics of the depurination.

It is biologically unreasonable to postulate different DNA contents in mouse thoracic duct B and T lymphocytes, yet, it has been shown

that statistically (68) significant differences in total O.D. can be found when they are stained together.

The emerging bimodality of the O.D. distribution in sets of peripheral blood lymphocytes from the guinea pig observed after two days of treatment with DNCB, is most likely to reflect a slight difference in sensitivity of cells from two differing functional states to the drug effect, and to hydrolysis in the Feulgen procedure.

No increases in total O.D. values were observed, and cell areas decrease together with the total optical density. There is no indication that transformation of cells occurs in proportions larger than 2%; the number of cells observed in each set is too small to ascertain whether a smaller proportion of cells might show transformation. In samples collected from the experiment with 5 FU treatment only, a very few isolated cells with tetraploid DNA values were observed.

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