Photometric Approaches in the Study of Insect Immune Cells

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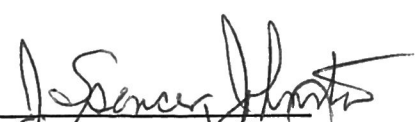
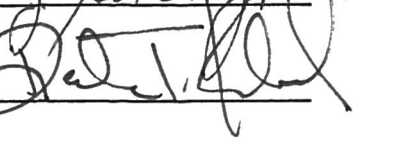
Fellows Advisor 

Honors Director 

Abstract

The study of insect immunology has been hampered by the limited quantity of material from any one individual. We have developed several techniques to make use of flow cytometry for the isolation and characterization of insect haemocytes. Flow cytometry was used to sort classes of *Heliothis virescens* (Fabricus) cells enriched for the larger, more differentiated blood cells which were then injected into mice for the production of monoclonal antibodies. Monoclonal antibodies are of interest because they are very specific, quantitative, and when bound to an appropriate fluorescent conjugated tag, readily recognized by the flow cytometer. We have also stained haemocyte DNA using mithromycin (and other DNA stains) to show the first known description of relative DNA amounts in insect blood cells. We find the cells to be 2C, 4C, and 8C with no higher ploidy or polyteny levels. Also shown is the capacity to score phagocytic cells and show the numbers and percents of cells that engulf one or more 1 micron fluorescent beads. We feel the production of monoclonal antibodies, plus the DNA and phagocytic studies described here, will provide useful new research tools for the study of insect haemocytes.

Photocytometric Approaches to the Study of Insect Immune Cells

Introduction

Mammalian immunogenetics has made great strides in the past 40 years. Immunoglobin has been characterized at the molecular genetics level, and different cell types have been characterized and their association described for different diseases. Control of disease has in a large part focused on modifying the relative numbers or types of cells through immunization and more recently through chemotherapy.

Progress in mammalian immunogenetics has been largely built upon the early isolation of specific cell types from large volumes of blood. These isolates were biochemically characterized and since 19xx used to produce the monoclonal antibodies that further characterized and identified immunological haplotypes. These advances have also been aided by flow cytometry, a modern instrument for counting and identifying cell types, which in combination with fluorescent conjugated antibodies has largely replaced microscopy for identification of cell types in immunology. The flow cytometer is fast, relatively reliable, and easily used - producing results largely independent of the immunological training of the operator.

Insect immunogenetics presents the same basic problems of mammalian systems, and is closely associated with insect pest problems in man. The problems are both agricultural and medical. The primary agricultural problem is associated with bio-control (parasitism of pest by other insects). It has been shown that failures in bio-control are largely due to the host's own immunological defenses. One of two closely related pest species - *Heliothis virescens* (Fabricus) - is controlled by the parasitic wasp *Campoletis sonorensis*. The other species *Helicoverpa zea* uses its immune system to encapsulate, and thus kill, the egg of the parasite. The molecular biology of this immune reaction is

described, and associated genes cloned; however, the specific immunological cells involved are unknown. The primary medical problems are parallel. That is, a species of insect will transmit to man a parasite, such as falciparium malaria, while a closely related species of insect will not transmit the disease because its immune system will not tolerate the parasite. And, while much is known about the transmission and spread of the various insect vectored parasitic diseases, little to nothing is known about the interaction of the parasites and specific insect immunocytes.

An overview of Insect haemocytes

The different cell types of *Heliothis virescens* (Fabricus).

The insect haemocyte cell types are varied in appearance and function from species to species. However, certain generalities can be made about the types and morphologies present. The following classifications are based on the Rowley and Ratcliffe (1981) scheme and include the cell types found in *Heliothis virescens* (Fabricus):

1) Prohaemocytes (PRs): These small, round cells are considered to be the stem cells from which certain other classes develop. They are usually more abundant in the larvae haemolymph than in adult.

2) Plasmatocytes (PLs): These phagocytic (Brehelin and Zachary, 1986) cells are round or spindle shaped, and are usually the most common class of circulating haemocyte. They have been found to be involved in encapsulation, nodule formation, and wound healing.

3) Granular cells (GRs): While some PLs appear granular in nature, this class is distinctive in that the cells are adherent by fine filopodia, but are rounded and phase bright *in vitro*.

4) Spherule cells (SPs): This class contains large spherical phase-bright inclusions, that stain for mucopolysaccharides.

5) Oenocytoids (OE): These large, transparent cells are considered to be fairly uncommon in circulation, and burst within minutes of being isolated from the insect.

The Origin of Haemocytes.

The presence and site of Haemopoietic organs (HPO) varies with an insects developmental stage, as well as phylogeny. HPOs can exist as small clusters of cells, or may be part of highly organized tissue. The HPOs of *Gryllus* and *Locusta* have been described by Haffmann *et al* (1979, review) and provide an example of exopterygotes. In the house cricket *Gryllus*, the highly organized tissues are found on either side of the dorsal vessel in the second and third abdominal segments. The organs are delimited by connective tissue and fibro-blast like cells, and the cortical region comprises a meshwork of reticular cells. Some of the progeny of dividing reticular cells become haemocytoblasts, and these divide to produce "isogenic cell islets", that include either PLs or GRs. Differentiated cells are found free in the lumen, and are assumed to pass freely into the haemolymph via the dorsal vessel. In *Locusta*, the HPO is not as well organized, and is found to be irregular accumulations of cells along the dorsal vessel.

Endopterygotes differ from exopterygotes in that the sites of haemopoiesis appear to be lost after the last molt. In *Euxoa declarata* (Hinks and Arnold, 1977), HPOs are associated with imaginal wing discs. The HPOs appear to atrophy after the six instar, and disappeared after the pupation. This process is common for many insects, and gives rise to an interesting puzzle: if the HPOs are no longer producing immune competent cells, how are these cells maintained?

Mitosis in Circulating Haemocytes.

The ability of circulating haemocytes to divide and increase their numbers is an important question which has yet to be fully answered. The general methods employed have been examination of fixed cells under the microscope to pulse labeling with [^{3}H]thymidine. In adult Orthoptera and Dictyoptera whose HPOs persist, mitosis of circulating haemocytes is rare, even after injection with bacteria (Ryan and Nicholas, 1972), and appears limited to the PLs. It is assumed that the replenishment and control of the haemocyte level is controlled by the HPO. In adult Lepidoptera and Diptera, in which HPOs disappear at pupation, haemocyte levels are presumably maintained by mitosis of the circulating haemocytes. Opinion differs; however, as to which cell classes undergo mitosis.

There are few similarities between mechanism of maintanence in the haemocyte. In general, most of the exopterygotes probably rely on their HPOs. When the immune system is challenged haemopoiesis is stimulated. In endopterygotes, in which the HPOs disappear, it does appear that circulating cells can divide and maintain the proper level of the population.

In Vitro Culture of Haemocytes.

Researchers have studied insect immune systems for over three decades, but the *in vitro* study of the immune cells has proven very difficult. The most difficult problem facing research in the field is the reactivity the immune cells posses. While methods of keeping collected cells intact have been developed, most attempts at cell culture have been disappointing at best.

Haemocytic defense Mechanisms.

Haemocytes are involved in many different functions, including the transport of lipids (Chino 1985), proteins (Geiger et al, 1977) and tyrosine (Post, 1972). In addition to those

functions, haemocytes are involved in blood coagulation, wound healing, formation of connective tissues, secretion of humoral antibacterial factors and cellular immune responses. Bacteria, viruses, and other small particles are removed by phagocytosis and encapsulation.

Phagocytosis is the major pathway by which small particles such as bacteria and viruses are internalized, and it is accomplished by both circulating haemocytes and sessile reticular cells. The quantitative work of Ratcliffe and Walters (1983) using various bacteria found that low doses of bacteria - less than 10^3 µl - were removed by phagocytosis. When the injected levels were increased, nodule formation was used to clear the haemolymph of the invading organism. The cells most associated with phagocytosis are the PLs, although GRs also appear to be phagocytic. The proportion of cells that are phagocytic is variable, and depends on the dose and type of particle used (Rabinovitch and de Stefano, 1970; Hanschke et al. 1980; Ratcliffe and Walters, 1983), the time at which the haemolymph is analyzed, and the prior experience of the insect.

When aggregates of haemocytes entrap particulate material nodules, are formed. Depending on the dose, the removal of unwanted bacteria can be very rapid (Gagen and Ratcliffe, 1976.) In larvae of Lepidoptera de-granulating GRs are found associated with live or dead bacteria within minutes of injection. The bacteria become trapped in the coagulum, and the PLs start to aggregate. The mixture of bacteria, GRs, and PLs, flatten and form a capsule-like structure which usually adheres to surfaces of tissues within the haemocoel (Ratcliffe and Gagen, 1976, 1977).

Encapsulation is the isolation, within many layers of haemocytes, of objects too big to be phagocytosed or trapped within nodules. The general structure of a capsule is an inner layer of rounded, melanized haemocytes that are surrounded by layers of flattened

interdigitated cells. There may also be another layer of rounded cells on the outer surface (Lackie *et al.*, 1985). Capsules are not all the same thickness, and vary between species depending on the number and proportions of haemocytes available (Gotz, 1986; Lackie *et al.*,1985).

Changes in the Haemocyte population.

The concentration and cell types in the haemolymph vary throughout an insects life, and many factors affect these concentrations. Factors such as development and reproductive status, wounds, or invasion by parasites all play a role in the status of the haemocytes. In general there is an increase in the cell number with instar. Arnold and Hinks (1976) found that between the second and sixth instar of *Euxoa declarata* the relative cell count more than tripled. As might also be expected, stress such as overcrowding, starvation, and non-physiological temperatures lower an insect's cell numbers and immunocompetence.

One of the most important functions of haemocytes is wound healing. Thus, wounding affects both the cell numbers and proportion of cell types. Harvey and Williams (1961) pricked brainless dispausing pupae of *Hyalophora cecropia* and found that the cell number increased 10 times within 30 minutes, and continued to increase with each successive sample for the next 6 days. Third-instar larvae of the tussock moth, *Orgya leucostigma*, were found to have an elevated cell count 4 hours after cuticle puncture. A more rapid increase in cell count occurred when the larvae were injected with saline, the number approximately doubling in 3 hours (Guzo and Stoltz, 1987). The collection of cell count data has to be coincident with strict controls to take into account cell counts that are increasing naturally.

Recognizing self vs. non-self.

Non-self recognition is one of the most important roles of cellular immunity. The mammalian basement membrane contains a network of non-fibrillar (type IV) collagen, to

which protoglycans and glycoproteins are attached (Timpl *et al.*, 1981). In insects, type IV collagen has been demonstrated in the basement region by various techniques including electron microscopy (Biasi and Piloto, 1976), biochemistry (Ashhurst and Bailey, 1980) and immunochemistry (Natzle *et al.*, 1982). Although the presence of glycosaminoglycans (GAGs) have been demonstrated (Yamada, 1983), very little is known about their use in non-self recognition.

There are several properties which seem to enhance the cellular response of the haemocytes. The net charge of the injected particle has a strong influence on the encapsulation process as shown by Vinson (1974) with *Heliothis* larvae, Dunphy and Nolan (1982) in *Choristoneura* larvae, and Lackie (1983) in adult *Schistoneura*. Encapsulation of agarose beads in *Schistoneura* did not occur until the charge of the beads were near neutrality, and further studies indicated that the charge of the immune cells themselves had some effect on their encapsulating ability. A stronger immune response appears to be elucidated by lipopolysaccharides and peptidoglycans. Lackie and Vasta (1988) have shown that the *in vivo* response of haemocytic encapsulation is influenced by the carbohydrate composition of foreign surfaces in *Periplanta*.

Materials and Methods

Insects.

The USDA stock of *Heliothis virescens* (Fabricus) corn earworm, used in these studies has been reared in Dr. Max Summers lab (TAMU) since 1989 at room temperature on Bioserve corn earworm food. Instar determinations were made on the basis of head width.

Monoclonal Preparations.

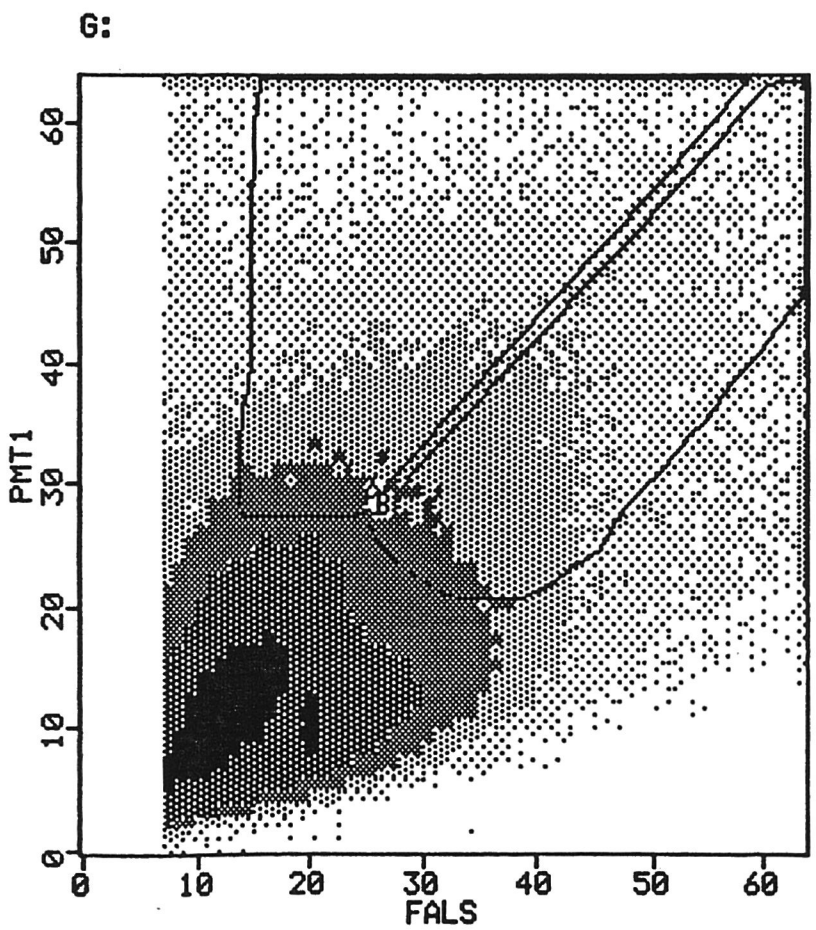
Fifth instar larvae of *Heliothis virescens* (Fabricus) were bled by cutting the abdominal proleg. Blood cells were collected in 0.7 ml of cold ACR solution (40 ml of 1M NaCl, 254.8 ml 5M KCl, 156.9 ml 0.5M EDTA, 94.2 ml 1M Citric acid, 271.3 ml 1M Na Citrate, 25 ml 1M KPO4. Filled to a final volume of 10L with filtered water and adjusted to a pH of 7.2-7.8 with NaOH) , and the collected cells sorted based on forward versus side scatter with ACR as the sheath fluid. We sorted blood cells into left and right sorts following criteria shown in Figure 1. The left and right sort areas made up about 24% of the total blood cell count.

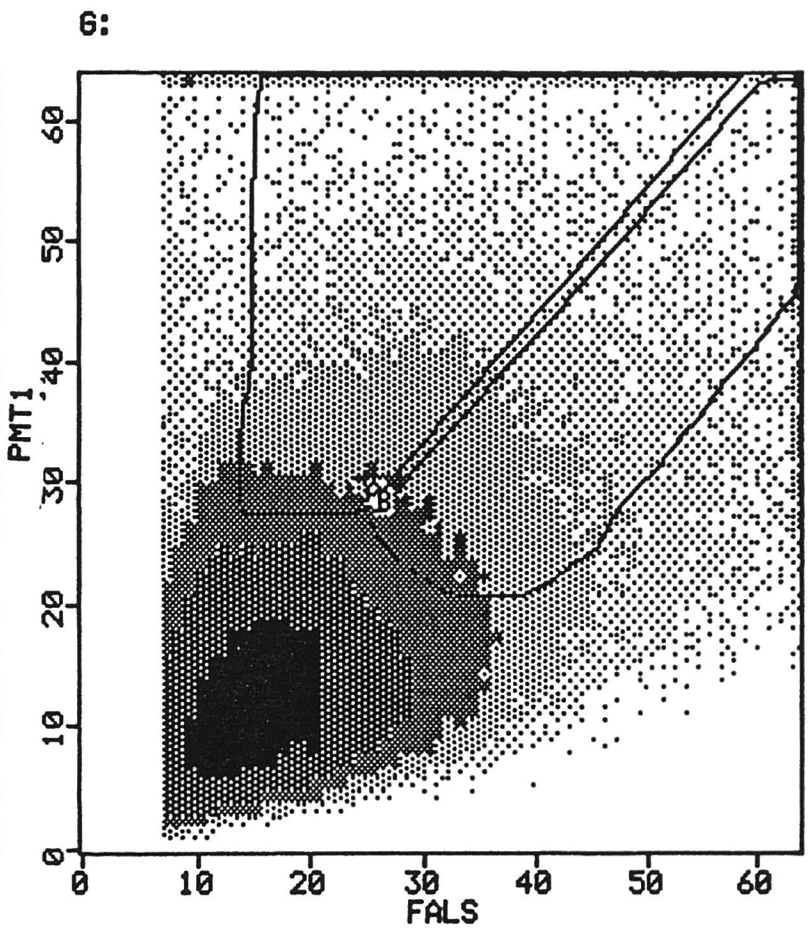
In order to provide material for injection into mice, the sorted cells were stored in horse serum (with 10% DMSO) at -80 °C. The cells were then washed in horse serum and injected intraperitoneally into BALB/c mice, using about 10^6 insect cells.

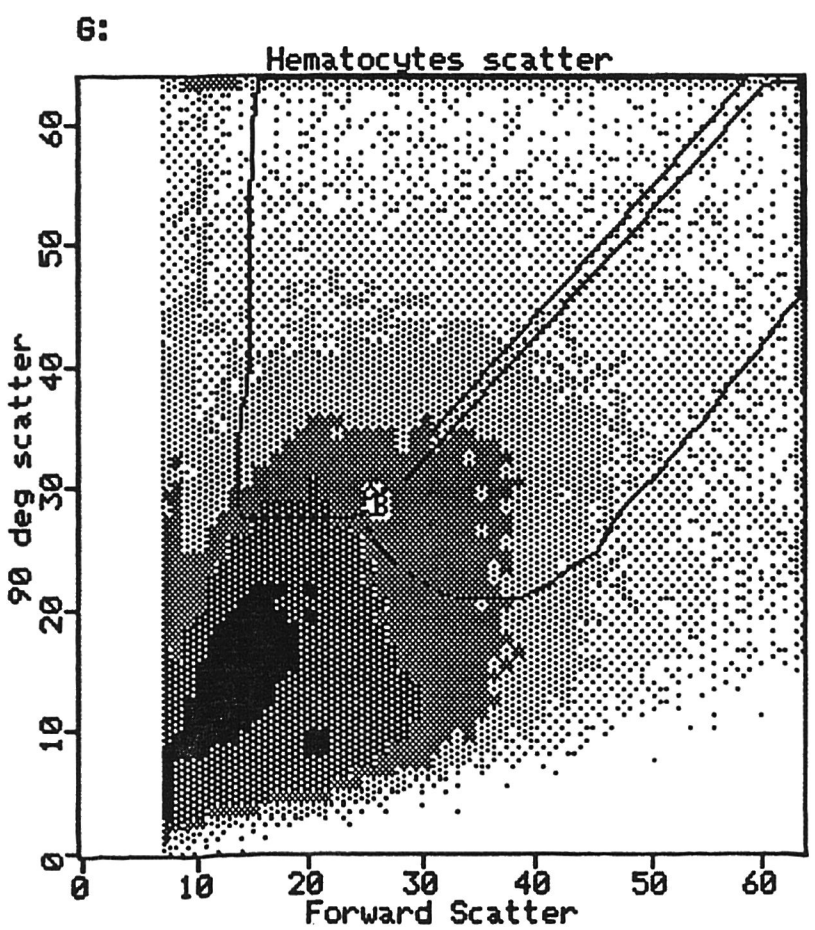
DNA and Bead Uptake Experiments.

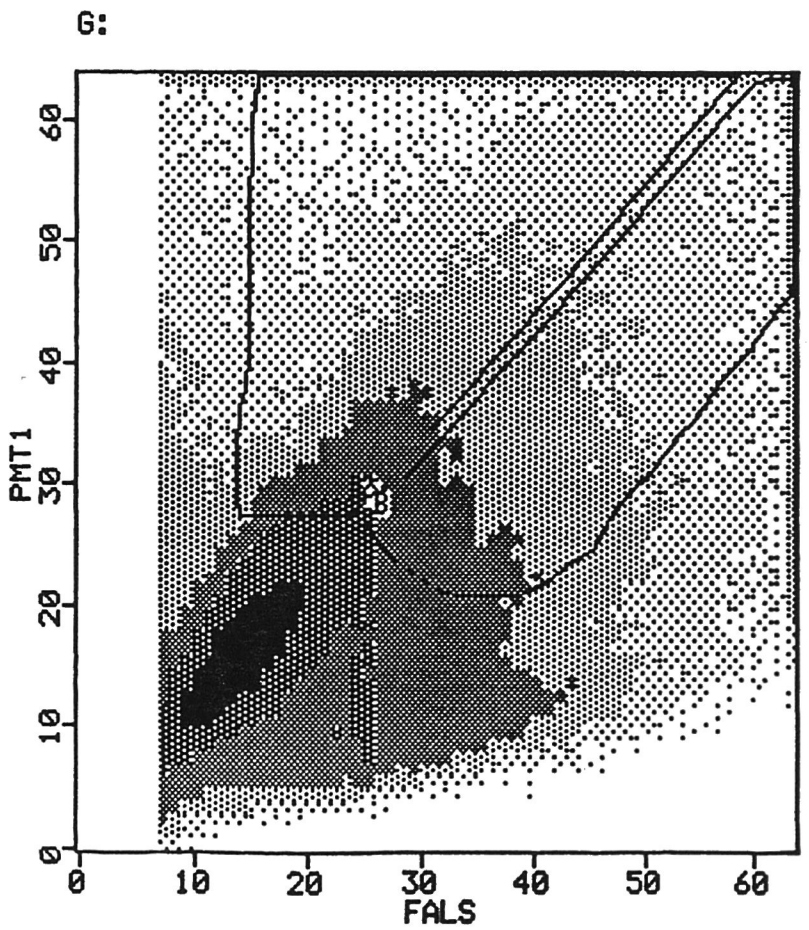
Fluorescently labeled bead solutions were made by mixing 200 µl of fluorescein bead solution (Molecular Probes, Inc. L-5282) (1 micron diameter, 2.5% solid, latex bead solution) in 1.5 ml of a phosphate buffer solution (PBS) (10 mM $NaH_2PO_4 \cdot H_2O$, 10mM $Na_2HPO_4 \cdot 7H_2O$, 140 mM NaCl, pH to 7.2)

Figure 1 - Sort Criteria for Monoclonal Injections









Injection: Fifth instar insects were immobilized with CO_2 gas, and injected dorsally between the body and head with ~30 µl of bead solution using a hypodermic needle.

Collection procedure with flow cytometry: At either 2 or 4 hours after injection, the insects were bled into 1ml of cold PBS, and the solution was centrifuged 3 minutes at 2500rpm. The supernatent was discarded, and the cells washed with 1ml of cold PBS and centrifuged as before. After discarding the supernatent the cells were resuspended in 1ml of glycol fix (1 M 2-Methyl-2,4-pentanediol in 50% ethanol/ 50% PBS). The cells were then run on an Epics elite flow cytometer with ACR solution and classes of cells were sorted with 488 nm laser light. The left and right sorts followed the criteria shown in Figure 2 (left sorter contained and two beaded cells, right sorter contained 6 or more beaded cells.)

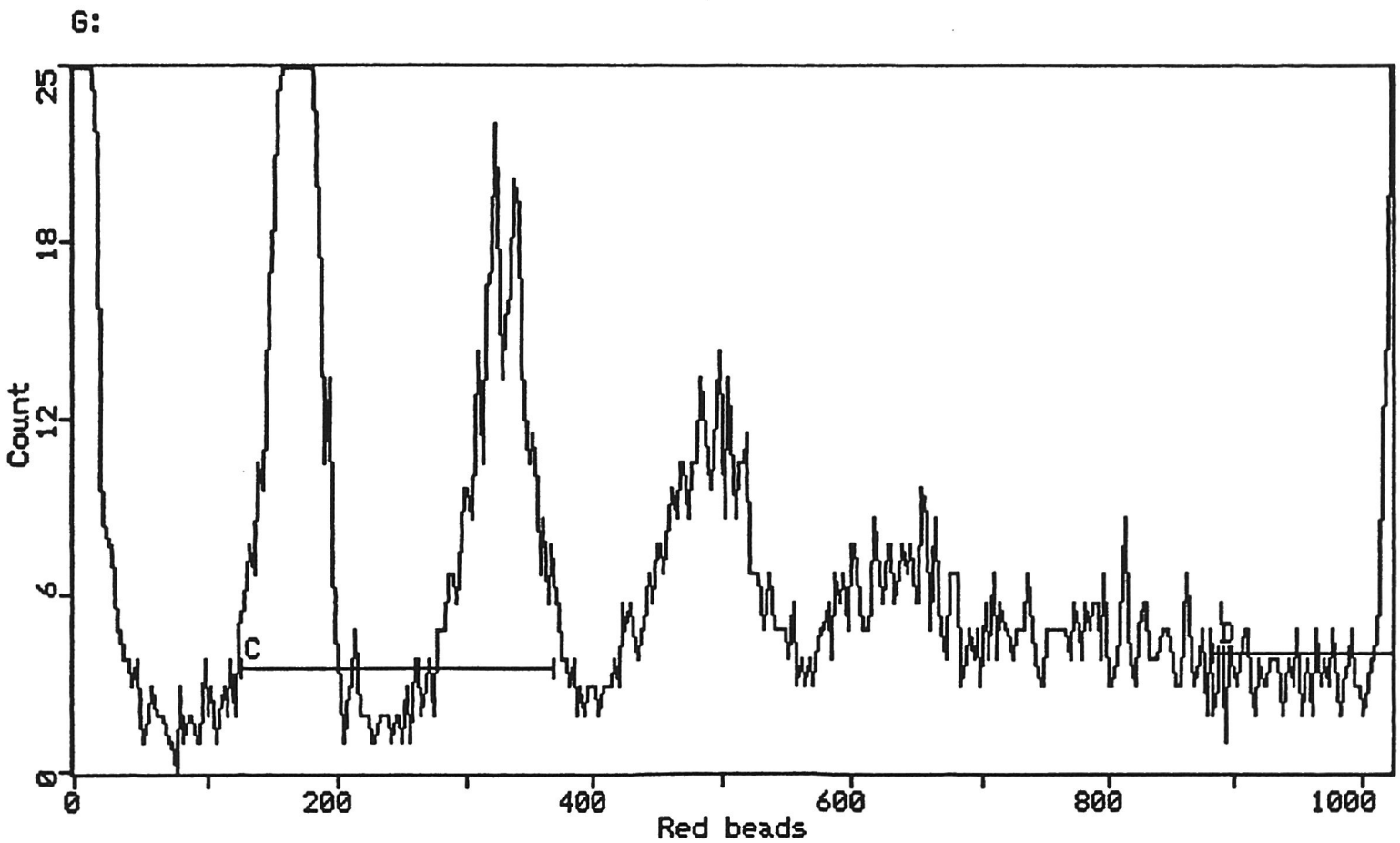
<u>DNA staining procedure.</u>

The cells were centrifuged as above, and resuspended in a mithramycin staining solution (100 µg/ml mithramycin, 15 mM $MgCl_2$, 25% ethanol) for 30 minutes. The samples were run on the flow cytometer and observed with 457 nm laser light. Cells fixed in 4% paraformaldehyde were stained with DAPI (4,6-Diamidino-2-phenylindole) (Sigma D1388) at 50 ppm in .1% Triton X. For vital staining, cells bled into ACR were stained with Hoechst 33342 (2'-[4-ethoxyphenol]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole) (Sigma B2264) at 50 ppm in ACR or with chromomycin A_3 (Sigma C2659) at 50 ppm in ACR.

<u>Microscopy procedure</u>.

Insects were injected as above, and bled into PBS solution, then the cells were transferred to a microscope slide and covered. The photographs are Leitz orthoplan (Figures 3 and 6) at 40X with 10X eyepiece for a total magnification of 400X.

Figure 2 - Sort Criteria for Bead Uptake



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SAMPLE NUMBER:					SAMPLE TIME: 18:14:09
COMMENTS	:				

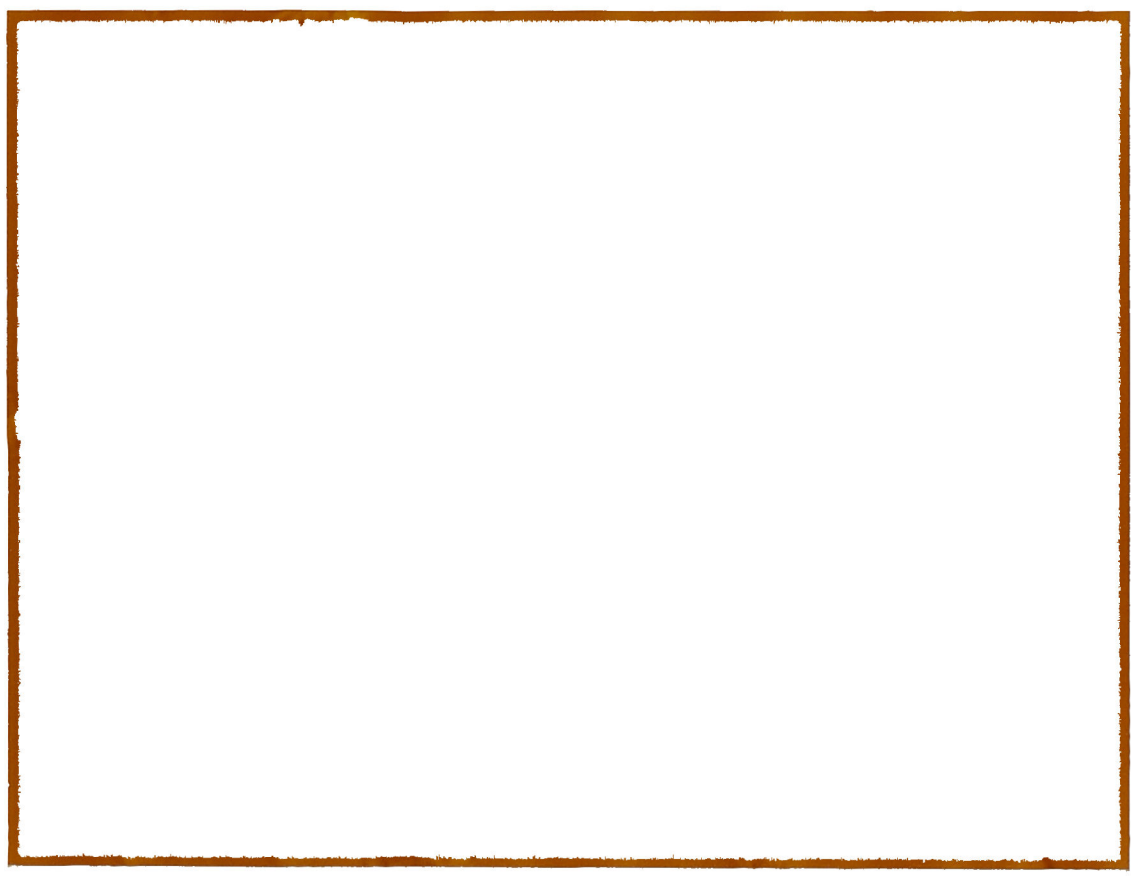
---------- STATISTICS ----------

SINGLE PARAMETER STATISTICS

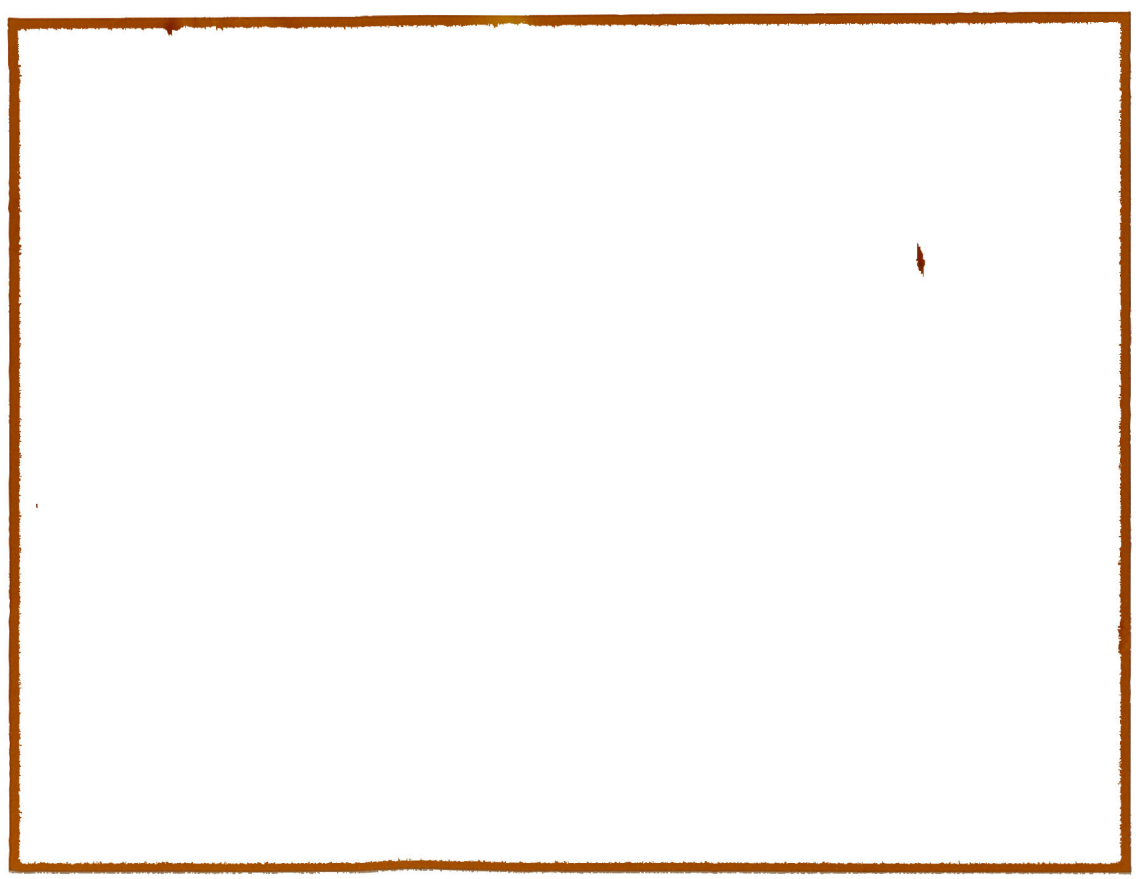
			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
C	9.7	2999	169	64	228.4	77.6	34.0	3.81	124	368
D	11.0	3410	1023	2786	1012.6	29.5	2.91	****	883	1023

C Low Channel = 124, High Channel = 368 C
D Low Channel = 883, High Channel = 1023 D

Figure 3 - Pictures of Bead uptake into Haemocytes



Several classes of cells with beads



Several cells forming a nodule around a large mass of beads.

Results

Sorting of regions for production of enriched samples for injection into mice is shown in Figure 1.

Table 1 - Sort region (A & B) Statistics.

Avg. # cells	St. Err	% A	St. Err.A	%B	St. Err.
257945	70.27	12.08	0.508	12.31	0.508

n= 20

The average run time was 16 minutes per individual. Clogging was a problem, and it was sometimes necessary to clean the sample tube of residue with bleach. However, clogging problems were considerably reduced by cleaning all equipment which came into contact with haemolymph with distilled water. To ensure that the regions selected were consistent with previous runs, minor adjustments to PMT1 and forward scatter were made as necessary to provide uniformity during each run. It is not known if the adjustments were necessitated by a difference in cell differentiation between samples, or by slight adjustments in cell size associated with osmolarity differences between blood and the ACR solution used to dilute the samples, but the adjustments were small (<5% of PMT voltage).

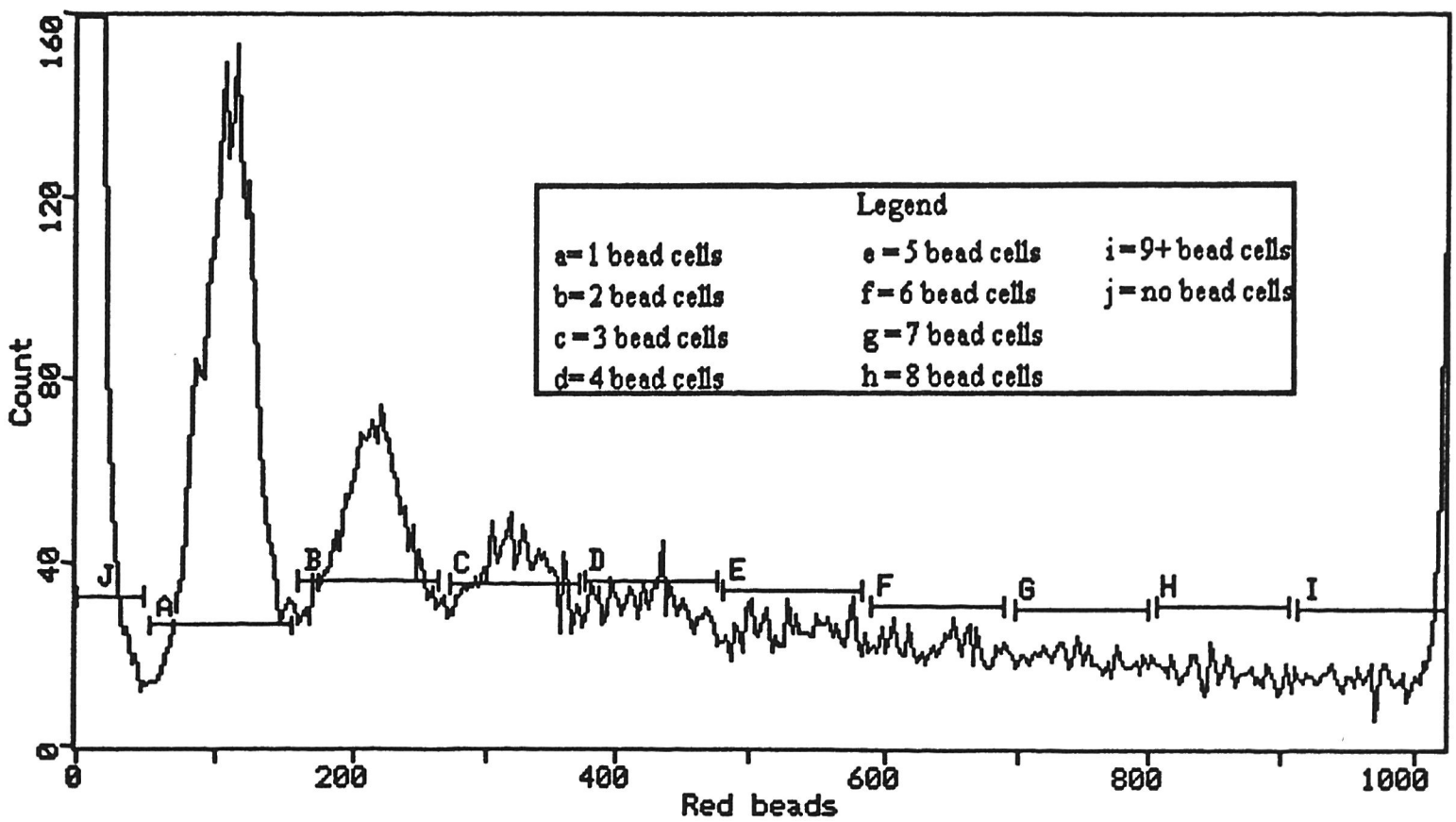
Uptake of beads.

The latex beads were injected into the larvae, and the larvae bled at 2-4 hour periods for the study of phagocytosis by cell types.

Latex bead engulfed in cells are shown in Figure 3.

Figure 4 shows the number of beads present in the cell versus the number of cells present.

Figure 4 - Percentage of Cells Engulfing Beads



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SAMPLE NUMBER	:	SAMPLE TIME	: 14:42:24
COMMENTS	:		

---------- STATISTICS ----------

SINGLE PARAMETER STATISTICS

			.....Peak.....		.....X Channel.....					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
A	8.8	7317	106	163	108.5	21.0	19.3	15.4	53	155
B	6.1	5094	222	80	213.2	26.4	12.4	9.24	160	264
C	4.6	3862	313	58	321.9	27.3	8.49	8.99	272	372
D	3.9	3210	436	48	425.5	28.7	6.76	1.72	376	477
E	3.1	2609	501	42	532.8	29.1	5.47	1.55	482	583
F	2.7	2256	608	34	639.9	29.4	4.59	0.683	590	691
G	2.4	1965	744	31	747.0	29.0	3.88	0.497	698	799
H	2.0	1640	860	27	853.5	29.1	3.41	0.464	805	906
I	19.6	16289	1023	14169	1017.3	19.6	1.92	****	913	1023
J	45.6	37944	2	7670	5.5	6.0	109.7	64.7	0	49

Table 2 - Summary of fluorescent beads present versus the percent of cells

# beads	% ttl
no beads	45.6
1	8.8
2	6.1
3	4.6
4	3.9
5	3.1
6	2.7
7	2.4
8	2.0
9 or more	19.6

DNA staining data

Proper handling of the haemocytes is the single most important step in the production of low cv DNA estimates. We have found that the cells remain intact if bled into either ACR or PBS. PBS is used to wash the cells, and remove the proteins in the haemolymph, while ACR in the sample and sheath maintains cell morphology while running the cells through a flow cytometer. Fixative solutions were not as successful. Several different techniques were tried, including Triton X and 4% paraformaldehyde, both of which resulted in the destruction of the cells. The glycol fix we used is only successful in PBS washed cells. The washing of the cells with PBS presumably removed the unwanted proteins which are in the haemolymph, and which denature in ethanol.

The study of DNA amount in haemocytes requires a stain which can penetrate the cells, and is specific for DNA. The first attempts at staining used vital stains such as Hoechst 33342 and chromomycin A_3 (both at 50ppm). In our trials the stains were not able to penetrate all the cell types, and showed poor specificity for DNA. Propidium Iodide (50 ppm) was also tried in cells fixed in glycol. Again the stain did not uniformly

penetrate the cells, and even with the addition of RNAse, uneven staining resulted in poor results (see Figure 5). Mithromycin staining of fixed cells solved both the problems of penetrance and specificity. Viewed under microscopy, the cell nuclei were stained with only light background in the cytoplasm (Figure 6). When these samples were run of the cytometer three distinct bands of DNA resulted (see Figure 7).

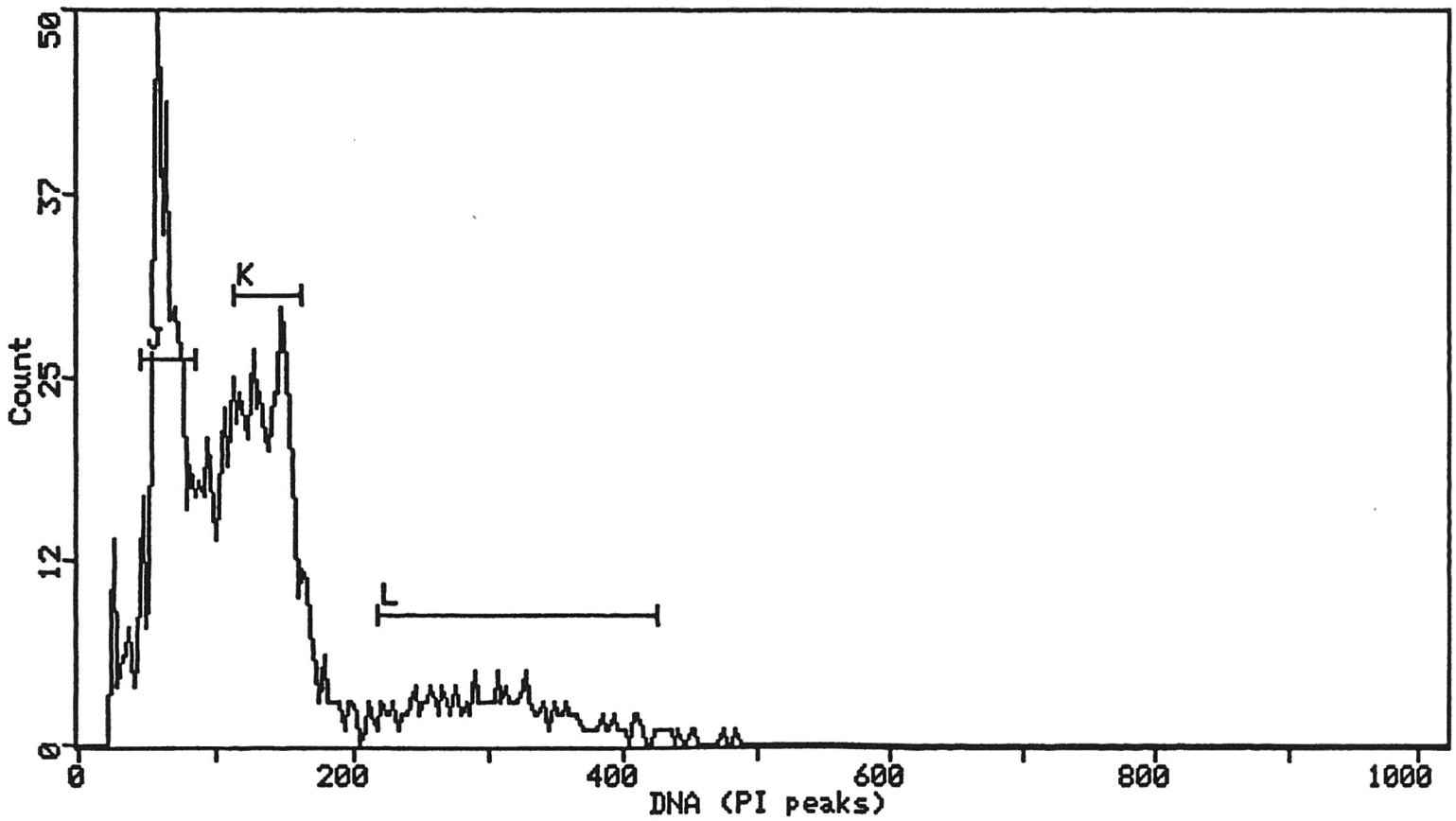
Mithromycin stained cells are shown in Figure 6.

Flow cytometer run data with mithromycin stained cells is shown in Figure 8.

Table 3 - Variations in Cellular DNA Amounts

2C % ttl	4C % ttl	8C % ttl	ratio 2C/4C	ratio 4C/8C
55.2	23.9	13.3	2.306	1.796

Figure 5 - Propidium Iodide Staining of Haemocyte Nuclei



SAMPLE INFO

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SAMPLE NUMBER:					SAMPLE TIME: 16:48:46
COMMENTS	:				

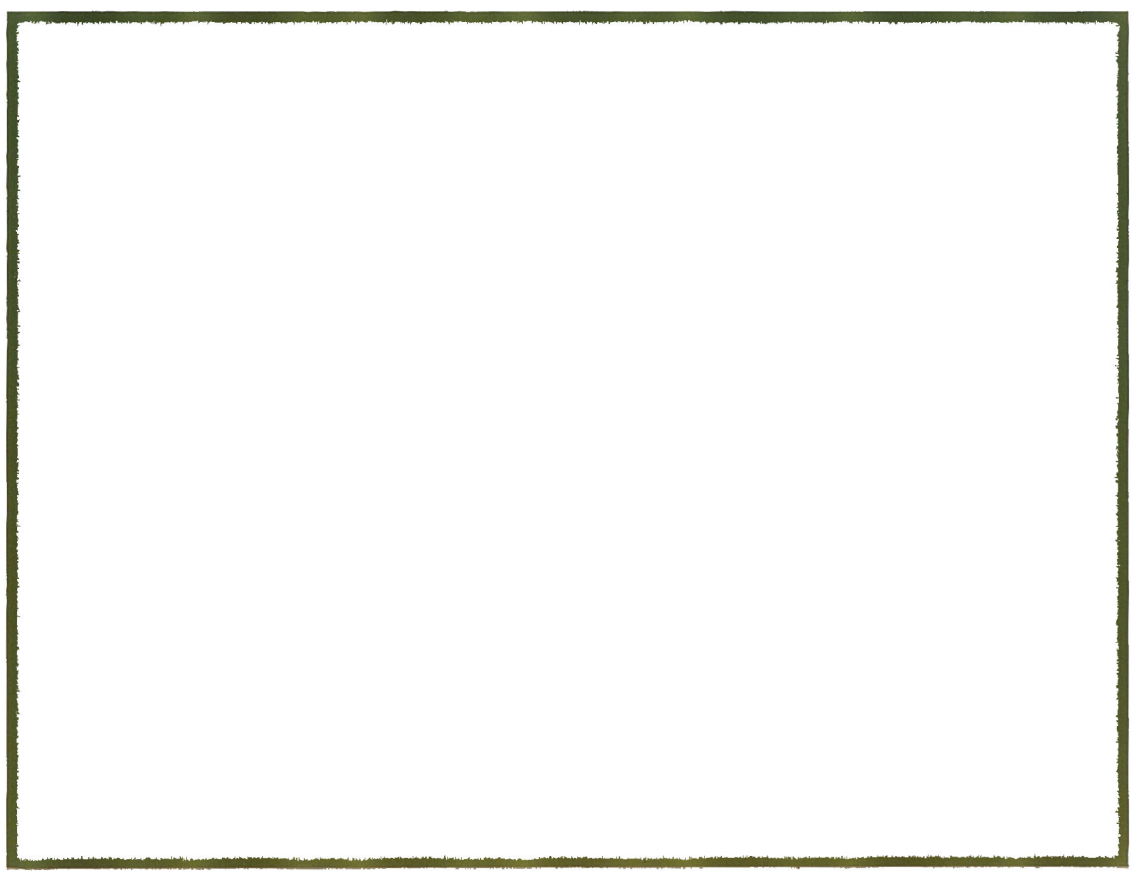
STATISTICS

SINGLE PARAMETER STATISTICS

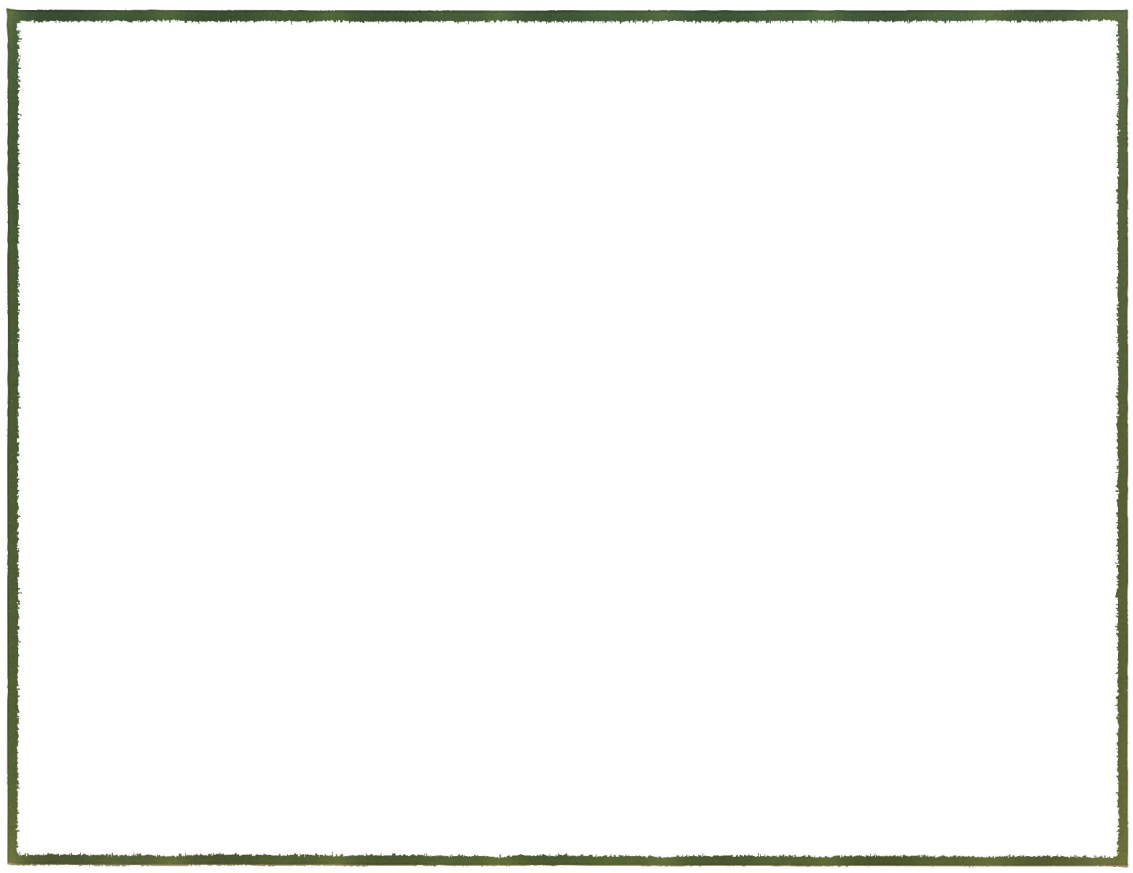
			Peak		X Channel					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
J	31.1	1129	57	162	64.2	9.7	15.1	1.38	44	84
K	30.9	1120	127	32	135.4	13.8	10.2	4.97	112	161
L	13.7	496	328	11	306.4	51.6	16.8	0.168	216	425

J Low Channel = 44, High Channel = 84 J
K Low Channel = 112, High Channel = 161 K
L Low Channel = 216, High Channel = 425 L

Figure 6 - Mithromycin Stained Haemocytes

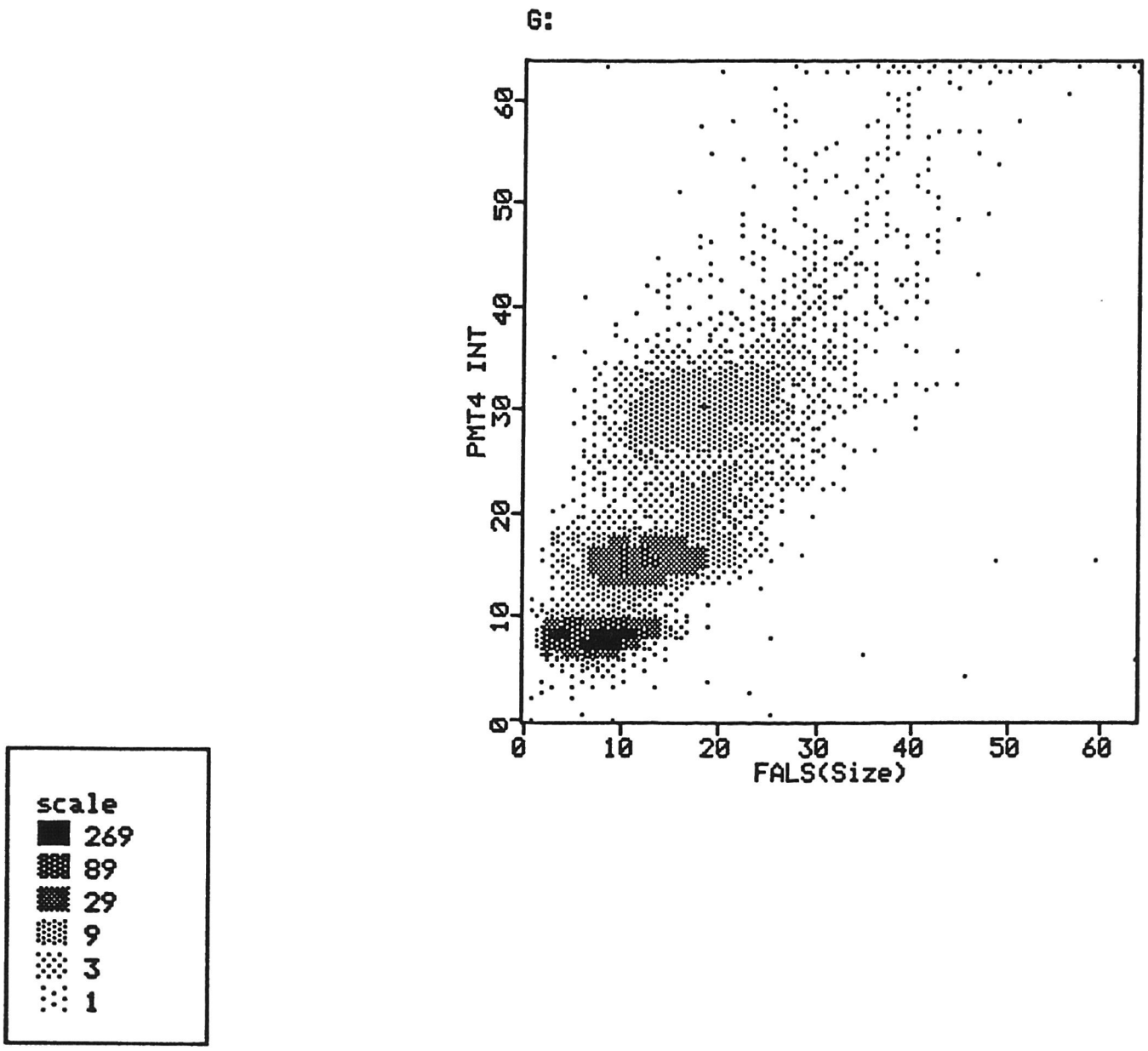


Fluorescently labeled DNA of several nuclei



Fluorescently labeled DNA of several nuclei and one engulfed bead

Figure 7 - Scattergram of Mithromycin Stained Haemocytes



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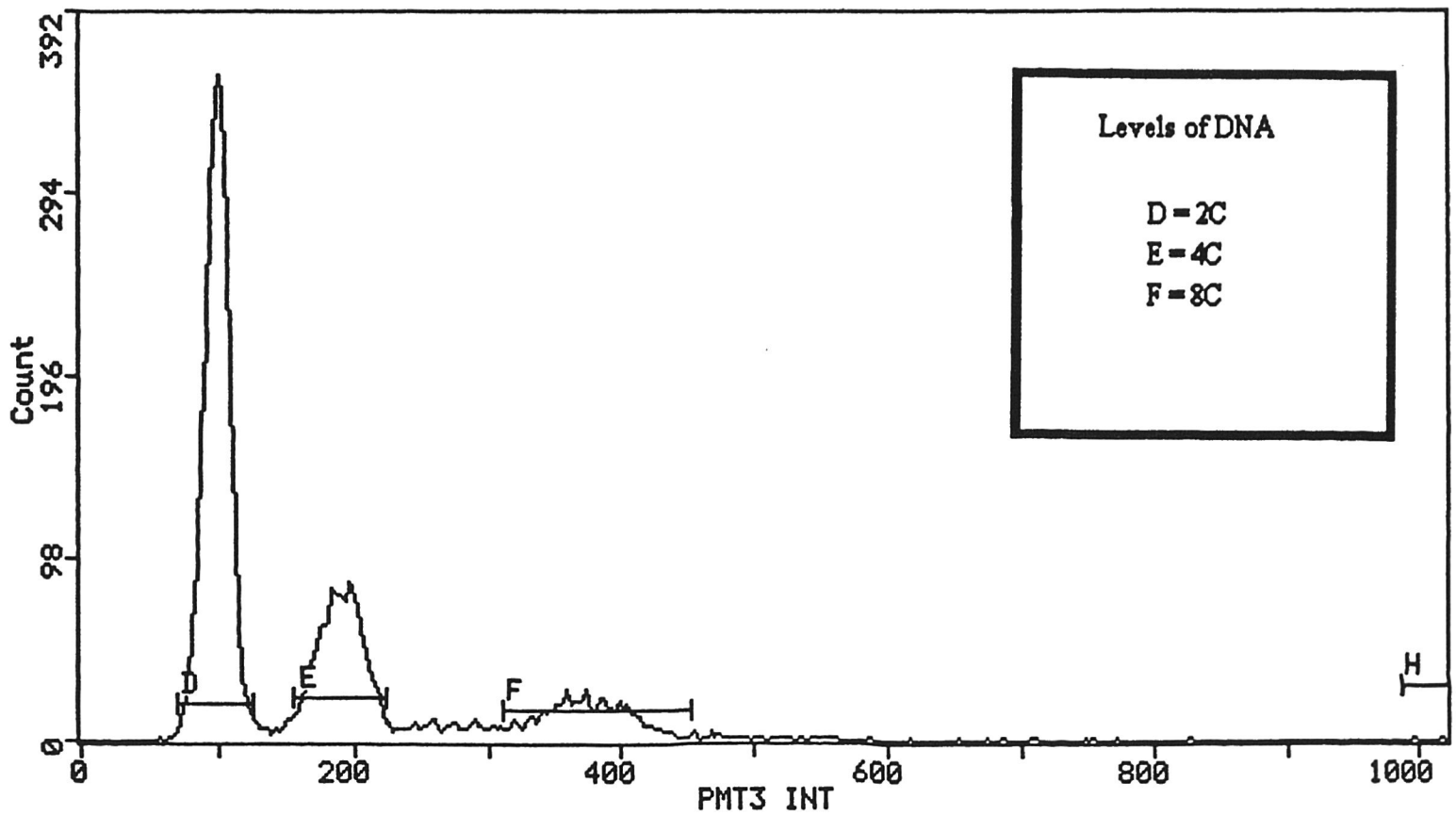
COMMENTS	:				

Figure 8 - Histogram of Mithromycin Stained Haemocytes



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PROTOCOL	:	h.virescence and beeds mithr			
DATA RATE	:	84	HIST COUNT :	14,506	LIST FILE : Fi001987.LMD
INSTRUMENT	:	Elite			PROT FILE : h0000037.PRO
SAMPLE NAME	:	Fifth Instar Control B			SAMPLE DATE: 29Mar94
SAMPLE NUMBER:					SAMPLE TIME: 11:15:31
COMMENTS	:				

---------- STATISTICS ----------

SINGLE PARAMETER STATISTICS

			......Peak......		.....X Channel.....					
ID	Pcnt	Area	Position	Height	Mean	SD	FullCV	HalfCV	Min	Max
D	55.2	8004	100	392	98.9	9.0	9.13	8.44	69	125
E	23.9	3469	195	102	190.0	15.3	8.04	7.61	155	224
F	13.3	1931	362	34	374.7	31.5	8.41	0.769	310	453
H	0.1	16	1023	10	1017.0	11.0	1.08	****	986	1023

D Low Channel = 69, High Channel = 125 D
E Low Channel = 155, High Channel = 224 E
F Low Channel = 310, High Channel = 453 F
H Low Channel = 986, High Channel = 1023 H

Discussion

The study of insect immunology has been hampered by the limited quantity of material from any one individual; and novel methodological approaches are needed to isolate and study the insect immune blood cells. Using techniques that make use of the analytical capacity of the flow cytometer we have developed several approaches for the study of the blood cells in *Heliothis virescens* (Fabricus) , and which can be readily adapted to other insect systems. The experience of mammalian researchers indicates that many cell types exist with identical morphology, but different functions. Presumably the same is true in insects. In mammals monoclonal antibodies have been an essential tool to further identify different cell types. And so a similar approach was begun in this study. Monoclonals are very specific, quantitative, and when bound to an appropriate fluorescent conjugated tag, readily recognized by the flow cytometer. As such, monoclonals seem ideal for insect studies as they will permit a relatively clean, fast isolation of insect cells.

Insect Immune Cell Sorting and Monoclonal antibody production.

The major advantage of the flow cytometer is its capacity to sort different cell types from a mixture. Using only the forward and 90 degree scatter parameters of newly collected live blood cells we have isolated two classes of differentiated cells and used these two sorted cell classes for BALB/c mouse injections. We have completed the first injections, and cells have been stored for the next two boosters. Once the course is complete, the mice will be sacrificed, and their spleens removed using proper sterile technique. Because the work is still in progress it is possible only to describe the process and its potential value.

Upon booster completion, the spleen cells will be released into a single-cell suspension, and are harvested using centrifugation. After washing, these spleen cells will be counted and grown on a medium commonly referred to as DME. The cells can then be grown in

bulk, and a portion stored in liquid nitrogen. The spleen cells are fused with myeloma cells to prepare hybridomas using polyethylene glycol (PEG), and the cells plated onto HAT plates. The cultured cells will be grown in wells with feeder cells, and after a week the cultured cells can be plated on a medium (other than HAT since all the parental myeloma cells will be dead.)

The screening assay will be the most important part in hybridoma production, and is the step where antibodies are selected for their specificity to a single cell line. The screening assay will be an ELISA screening, and the hybridomas will be tested first against whole insect blood to check for reactivity; and then the positive isolates will be further screened against sorted cell types to test for cell specificity.

Once positive, cell specific monoclonal antibodies are discovered, the corresponding hybrid cells are grown in soft agar. The hybrid cells can be stored in liquid nitrogen. The antibodies produced by the hybrid cells can then be used with the immunofluorescence to mark immune cells for easy screening and characterization using flow cytometry.

Eventually, the hope is to submit sorted insect cells to a variety of biochemical and molecular analyses to compare and contrast the different cell functions. This study is therefore the first step in a long, very promising process.

<u>DNA Content Determination and Cell Cycle Analysis.</u>

Little is known about the life cycle of haemopoietic cells. In *Heliothis virescens* (Fabricus) the haemopoietic organ disappears after pupation, and yet there is still a functioning immune system in the adult. One possibility is that all of the cells necessary for the insects immune system function are replicated before pupation in the HPO. Another possibility is that the circulating immune cells can themselves divide and/or differentiate as needed by the adult. We were not successful in staining insect immune cells with existing mammalian vital staining methods. Staining the DNA with mithromycin

(as developed here) has shown us that there are three unique levels of DNA in the cells, which correspond to 2C, 4C, 8C. While the DNA analysis does not show that the cells are replicating in the blood, it has shown that the insect immune cells can be quantitized based on DNA content. As expected the high ploidy numbers corresponded to large cells as measured by forward scatter . Further, it shows that two fold ploidy "polyteny" is common, and may (with additional effort) provide a further tool for description and isolation of unique classes and types of haemocytes. Lastly, high ploidy levels (high polyteny) typical of some insect tissues are absent in insect blood. To more directly study DNA replication, future studies can make use of our technique by modifying it for replication specific fluorescent markers which recognize incorporated BrdU (Gratzner et al, 1975).

<u>Isolation based on Phagocytosis.</u>

One of the major functions of the insect haemolymph is the removal of small particles by phagocytosis or encapsulation. By injecting *Heliothis virescens* (Fabricus) larvae with 1 micron fluorescent latex beads we were able to simulate an infection in the larvae. The beads were then used as a marker to compare different classes of cells based on bead uptake (phagocytosis). While this technique alone cannot be used to give clean isolates, it does allow us to study a crucial cell function. The number of beads engulfed ranged from one to more than eight, and in general larger cells engulfed more beads. Approximately xx% of the cells engulf beads, the remainder engulf no beads within four hours of injection. Since there is an association of size in both DNA content and beads engulfed it will be of interest in future studies to compare phagocytic capacity to DNA content. At the same time, as monoclonals are developed it will be of interest to compare monoclonal binding to ploidy level and/or phagocytic capacity.

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Appendix - Flow Cytometer Settings

PROTOCOL: IMUNNOSORT .37 WATTS BLUE

Sensors

	FS	PMT1	PMT2	PMT3	PMT4
Volts	200	279	0	0	400
IGain	7.5	2.0	1.0	2.0	7.5
PGain			1.0	1.0	5.0

Discriminators

Chan#	Parameters
110	FALS
OFF	PMT1
OFF	Green beads
OFF	PMT3
OFF	Red beads
OFF	PMT1 LOG
----	TIME

Fluorescence Compensation

Signal out (Y) = Y - %X

Y \ X	PMT2	PMT3	PMT4
PMT2		0.0	5.0
PMT3	0.0		0.0
PMT4	15.0	0.0	

Fluidics (psi)

Sample	Sheath
11.40	12.00

Listmode Gate OFF

Miscellaneous

Bandwidth LOW

Laser(s)

	Req-mW	Amps	nm
#1	15	1.98	488
#2	10	0.00	633

OS File Name: Im000033.PRO Date Created: 01Oct93 Date Modified: 01Oct93

HISTOGRAMS (X/Y)		SAVE	DISPLAY	STOP @
1: Unnamed		No	Yes	No
SCALE	: AutoScale			
PARAMETERS	: FALS / PMT1			
2: Unnamed		No	Yes	No
SCALE	: AutoScale			
PARAMETERS	: FALS / PMT1 LOG			

FUNCTIONS

Gating:	ON	AutoAnalysis:	OFF
StopTime:	00:20:00	SortSettings:	Send
Listmode:	NoSave	CytoSettings:	Send

PARAMETERS	NAME	NO.	LOG
FS	FALS	0	:
PMT1	PMT1	1	:
PMT1 LOG	PMT1 LOG	5	4
PMT2	Green beads	2	:
PMT3	PMT3	3	:
PMT4	Red beads	4	:
TIME	TIME	15	:

CYTOMETER SETTINGS: DNA/BEAD UPTAKE IN HELIOTHUS V.

Sensors

	FS	PMT1	PMT2	PMT3	PMT4
Volts	173	246	554	530	580
IGain	15.0	10.0	30.0	15.0	7.5
PGain			1.0	1.0	1.0

Discriminators

Chan#	Parameters
OFF	FALS
OFF	PMT1
OFF	Green beads
OFF	orange bead
80	DNA (PI pea
OFF	PMT1 LOG
----	TIME

Fluorescence Compensation

Signal out (Y) = Y - %X

Y \ X	PMT2	PMT3	PMT4
PMT2		0.0	0.0
PMT3	0.0		0.0
PMT4	0.0	0.0	

Fluidics (psi)

Sample	Sheath
11.31	12.00

Listmode Gate

OFF

Miscellaneous

Bandwidth LOW

Laser(s)

	Req-mW	Amps	nm
#1	15	2.00	488
#2	10	0.00	633

PROTOCOL: PRE MITHR SORT

Sensors

	FS	PMT1	PMT2	PMT3	PMT4
Volts	153	283	0	0	740
IGain	5.0	5.0	7.5	7.5	10.0
PGain			1.0	1.0	7.5

Discriminators

Chan#	Parameters
100	FALS
OFF	PMT1
OFF	Green beads
OFF	PMT3
OFF	Red beads
OFF	PMT1 LOG
----	TIME

Fluorescence Compensation

Signal out (Y) = Y - %X

Y \ X	PMT2	PMT3	PMT4
PMT2		0.0	0.0
PMT3	0.0		0.0
PMT4	0.0	0.0	

Fluidics (psi)

Sample	Sheath
11.35	12.00

Listmode Gate OFF

Miscellaneous

Bandwidth LOW

Laser(s)

	Req-mW	Amps	nm
#1	15	1.98	488
#2	10	0.00	633

OS File Name: Pr000038.PRO Date Created: 29Mar94 Date Modified: 08Apr94

HISTOGRAMS (X/Y)	SAVE	DISPLAY	STOP @
1: Unnamed	No	Yes	No
SCALE : AutoScale			
PARAMETERS : FALS / PMT1			
3: Unnamed	No	Yes	No
SCALE : AutoScale			
PARAMETERS : FALS / Red beads			
4: Unnamed	No	Yes	No
SCALE : 25			
PARAMETERS : Red beads			
5: Unnamed	No	Yes	No
SCALE : AutoScale			
PARAMETERS : FALS			
6: Unnamed	No	Yes	No
SCALE : AutoScale			
PARAMETERS : PMT1			
7: Unnamed	No	Yes	No
SCALE : AutoScale			
PARAMETERS : FALS / PMT1			
8: Unnamed	No	Yes	No
SCALE : AutoScale			
PARAMETERS : FALS / PMT1			

FUNCTIONS

Gating:	ON	AutoAnalysis:	OFF
StopTime:	00:20:00	SortSettings:	Send
Listmode:	NoSave	CytoSettings:	Send

CYTOMETER SETTINGS: H.VIRESCENCE AND BEEDS MITHR

Sensors

	FS	PMT1	PMT2	PMT3	PMT4
Volts	520	380	850	0	990
IGain	20.0	20.0	5.0	1.0	30.0
PGain			1.0	1.0	7.5

Discriminators

Chan#	Parameters
100	FALS(Size)
OFF	90LS(Int+Ex
OFF	PMT4 PEAK
OFF	PMT4 INT
OFF	PMT3 INT
OFF	PMT2 INT
----	TIME

Fluorescence Compensation

Signal out (Y) = Y - %X

Y \ X	PMT2	PMT3	PMT4
PMT2		0.0	50.0
PMT3	0.0		0.0
PMT4	60.0	0.0	

Fluidics (psi)

Sample	Sheath
11.34	12.00

Listmode Gate OFF

Miscellaneous

Bandwidth LOW

Laser(s)

	Req-mW	Amps	nm
#1	0	1.98	488
#2	10	0.00	633