



B-600-355-78

R&S 102275

Splenic Lymphocyte Transformation in Culture as a Tool
for Immunotoxicologic Evaluation of Chemicals

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636-1089

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JUL 16 1980

SPLENIC LYMPHOCYTE TRANSFORMATION IN CULTURE
AS A TOOL FOR IMMUNOTOXICOLOGIC EVALUATION OF CHEMICALS

R&S 102276

September 22, 1978

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ABSTRACT

Splenic cells from mouse were cultured in microplates and the uptake of ^3H -thymidine determined to evaluate the lymphocytic transformation in short term (64-68 hrs) cultures. The cells undergo spontaneous blast formation as well as selective activation or subpopulation of lymphocytes by addition of phyto mitogens in culture media. The two phyto mitogens used in these studies were phytohemagglutinin (PHA) and pokeweed mitogen (PWM), which are known to be selective for thymus-dependent (T-lymphocytes) and thymus-independent (B-cells) lymphocytes from mouse spleen, respectively. The suitability of these cultures for immunotoxicologic studies involving various chemicals was evaluated. The blast formation of lymphocytes from mouse spleens decreased considerably with age in growing animals. Addition of small amounts of either mouse serum or liver homogenate significantly inhibited the thymidine uptake by cells in culture. Microsomes isolated from liver when added to these cultures had only a little influence on thymidine uptake. Exposure of male mice to different chemicals altered the transformation characteristics of their splenic lymphocytes. Addition of chemicals directly to lymphocyte cultures also caused different effects indicating that some of the effects can be caused directly on the cells. Based on these studies it is suggested that lymphocyte cultures from spleens can be utilized for toxicity evaluation of chemicals on immune functions of the organism. The results can be used to predict immunosuppression or

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immunostimulation potential of different chemicals. Further studies with a large number of chemicals will help ascertain the mechanisms and implications of the effects seen in lymphocyte cultures.

INTRODUCTION

Lymphoid organs and lymphocytes have been known to be intimately involved with different immune mechanisms in the body. Both cell mediated and humoral type immune responses are mediated through different types of lymphocytes. A general plan by which lymphocytes may acquire their differential and selective action is shown in Fig. 1. The population of peripheral lymphocytes consist of at least two different types of cells, i.e., T-cells that are involved in cell mediated and delayed type of immunity, and B-cells that are implicated in the secretory process of antibodies.

All lymphocytes originate in the bone marrow of higher organisms. Some of these cells migrate to the thymus, usually early in life, and become thymus dependent or "T"-lymphocytes. These cells proliferate into effector cells or memory cells. They are also involved in the production of various factors (i.e. lymphokines) that regulate the activity of other cells (e.g. B-cells or macrophages) and also have important biological actions. The outer membrane of T-lymphocytes possess a small amount of immunoglobulin molecules. B-cells, also originating in the bone marrow, are differentiated elsewhere in the body. In birds, this differentiation takes place in the lymphoid follicles located near the Bursa of Fabricius. In mammals, fetal liver and/or other lymphoid organs associated with the digestive tract may be involved in this differentiation. The surface of B-cells

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is covered with specific receptors or microglobulins that may be specific for the production of different immunoglobulins. These cells are the precursors of plasma cells that produce antibodies. (For more information see Hobart and McConnell, 1975).

In 1960, Nowell found that plant lectins such as phytohemagglutinin (PHA, a lectin derived from Phaseolus vulgaris) stimulates lymphocytes in vitro in a manner that is morphologically and biochemically similar to the responses induced by antigenic stimulation in vivo. Douglas et al., (1967) reported that another phytomitogen from pokeweed (PWM, obtained from Phytolacca americana) stimulated a significant number of cells to develop structural characteristics of the plasma cell line in vivo. A number of other chemicals that stimulate the lymphocyte responses include bacterial lipopolysaccharide (LPS, from E. Coli), concanavalin A (Con A) and antilymphocytic serum (ALS) (Andersson et al., 1971).

The fact that the lymphocytes can be selectively stimulated by different mitogens provided a tool for studying the subpopulation of these cells in vitro. Almost simultaneously, several workers reported that PHA was selective for mammalian T-cells whereas PWM induced mitosis in B-lymphocytes (Janossy and Greaves, 1971; Stockman et al., 1971). Some of these findings were made possible by the techniques that utilized DNA synthesis in cells as a measure of their mitotic activity. In short term cultures, the mitotic activity is exhibited by the formation

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of blast cells (hence these chemicals are also referred to as blastogens) and can be readily estimated by the incorporation of a labeled DNA base (e.g. ^3H -thymidine).

The evaluation of lymphocyte populations in vitro was suggested for monitoring the immunologic competence of lymphocytes from patients with various immunologic disorders and those undergoing immunosuppressive therapy (Oppenheim, 1968). It has also been used to describe the hypersensitivity reactions in responsive individuals (Halpern, 1972), and also to evaluate the immunosuppressive potential of environmental chemicals (Nahas et al., 1974; Vos and Moore, 1974; Caworski and Sharma, 1977).

This report describes a method that has been developed on the basis of those mentioned above and can be used as a routine test for immunologic evaluation in long or short term toxicity studies. The method can also be used for in vitro addition of chemicals or their metabolites to the cultures if a direct effect on the lymphocytes is to be evaluated.

METHODS

Cells and Materials. The spleen is a convenient source of lymphocytes for culturing from animals exposed to a test chemical. If a test needs to be performed in the interim periods, it can also be performed on whole blood samples (Park and Good, 1972). The presence of erythrocytes does not interfere with the assay and the blood samples can be used directly without any processing. If necessary, blood can be hemolyzed by diluting in a hypotonic saline solution and lymphocytes separated immediately by centrifugation. In the case of spleen, the organ is taken out aseptically from animals (decapitation is satisfactory) and stored in a sterilized physiological saline solution until the lymphocytes are isolated. It is suggested that the spleen not be allowed to sit for more than 2 hours for maximum viability of the cells. The spleen can be weighed if desired and rinsed in sterilized saline before use.

Other materials required for this method are: sterilized physiological saline solution, a set of sterilized forceps, supply of sterilized culture tubes, culture medium (RPMI 1640, supplemented just prior to use with 10% fetal calf serum, inactivated by heating at 56°C for 1/2 to 1 hour, penicillin 100 units/ml, streptomycin 0.1 mg/ml and L-glutamine 0.3 mg/ml), phytohemagglutinins (see below), microculture plates and covers (Falcon 3040), Eppendorf or a similar pipeting system with sterilized tips.

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The method described below is the one typically employed in our studies. The animals used in the following studies were male CD-1 mice obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.).

Preparation of Lymphocyte Suspensions. The whole spleen of a mouse or part of the spleen of other laboratory animals (usually 0.1-0.2 g tissue provides enough lymphocytes) was rinsed in sterilized saline solution and placed in a petri dish containing 5 ml physiological saline. The tissue was mashed with forceps until most of it was disrupted. The cell suspension was mixed well (by flushing through an 18-gauge needle with a syringe) and passed through a series of hypodermic needles (using a syringe and 18, 21 and 26 gauge needles in succession) to separate the unbroken tissue and cell aggregates. The suspension was finally transferred to a glass culture tube and allowed to stand for nearly 10 minutes to allow the cell aggregates to settle or adhere to the sides. Using a disposable transfer pipet, the suspension was transferred to a different culture tube leaving the bottom portion (ca 0.5 ml) behind.

The suspension (nearly 400 g) was then centrifuged at room temperature for 15 minutes. The supernatant was removed by suction and 3-4 ml of culture medium was added immediately over the cell pellet. The cells were suspended in the medium using a Vortex mixer. After allowing to stand for a few minutes, the cell suspension was transferred to another tube leaving the aggregated clumps behind.

It was considered desirable to process only 16-20 samples simultaneously and no more than 32 samples were processed at any one time so the process could be completed in one-half day.

An aliquot of the cell suspension was removed and the concentration of lymphocytes determined using an electronic counter. The red cells were lysed before counting. The viability of the cells was checked occasionally by adding a little trypan blue to an aliquot of cell suspension and the cells counted in a hemocytometer. The viability in our experience was always better than 95% and was not routinely checked. The original suspension was then adjusted to provide approximately 8×10^6 cells/ml. The cells were counted again after
ation and culturing to check the final concentration.

Lymphocyte Cultures. Aliquots of the above suspension, 50 μ l representing approximately 400,000 lymphocytes, were placed in each well of a microculture plate. The cultures were conducted either in triplicate or quadruplicate for each animal or addition of a mitogen, and thus 8 animals were accommodated in each culture plate. An arrangement of plating scheme is shown in Figure 2. In order to keep a record of culture numbers, a diagram was prepared on paper and labeled accordingly. The lymphocytes were allowed to settle on the bottom before any other additions.

After all samples were plated, 50 μ l of culture medium was added to each well. In cases when in vitro addition of a test chemical or other

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substance (e.g. liver supernatant) was desired, medium containing the appropriate chemical was added. Usually liver homogenate or serum constituted less than 25% of the medium (12.5 μ l) and the mixture added instead. Subsequently, an additional 50 μ l of either medium alone or medium containing PHA or PWM (see below) was added and the plates covered and incubated for 48-50 hrs. at 37°C in a humidified 5% CO₂ in air atmosphere.

The mitogens, PHA and PWM (obtained from Grand Island Biologicals, Grand Island, N.Y.) were reconstituted to 10 ml and portions diluted in medium to provide 0.5 μ l in each culture well. This provided optimum concentrations of lectins (about 2.5 μ l/ml in final incubation medium).

After the above incubation, the cultures were spiked with 50 μ l of medium containing 0.5 μ Ci of ³H(methyl)-thymidine (New England Nuclear, Boston, Mass.). The plates were reincubated for an additional 16 hours. The cells were then harvested using a Model ML2V Cell Harvester (Brandel, Rockville, Md.) and collected on Reeve Angel 934AH glass fiber discs. The cells were vigorously washed with saline. Precipitation by trichloroacetic acid (TCA) and repeated washing of cells with 3% TCA had no influence on the thymidine uptake; thus, the cells were washed with saline rather than TCA. The glass fiber discs, nearly dry because of vacuum suction in the harvester, were directly placed in regular scintillation vials. Scintillation fluid (Aquasol, New England Nuclear, Boston, Mass.), 10 ml, was added to each vial and the solution was well shaken. These vials were counted in a Mark III Scintillation Counter

(Searle Analytics, Inc.) for one minute each; the counts were converted to disintegrations per minute (dpm) using a standard quench curve. The dpm were averaged for replicate samples and adjusted for 10^6 cells in culture. The stimulation index for PHA or PWM was determined by dividing the dpm for cultures with respective mitogen by dpm from the similar culture containing no mitogen.

Whenever necessary, values from different animals in a group were averaged and the results were expressed as mean and its standard error.

RESULTS AND DISCUSSION

The purpose of this report is to describe and evaluate a method utilizing lymphocyte transformation (blast formation) in a culture system. The method used above has been modified primarily from that described by Janossy and Greaves (1971) and adapted for automated harvesting of cells. Many of the parameters have been evaluated previously and were either used or optimized from the information available. Generally the factors that may be of importance in evaluation of toxicity of chemicals were further examined.

Determination of ^3H -Thymidine Uptake. The cells were harvested on a mechanical harvester and were vigorously washed using saline as a wash solution. Whether this procedure disrupts cells so that only DNA bound thymidine (and not the soluble pool) is determined was questioned. In one of the experiments the cells were precipitated by addition of 7% trichloroacetic acid (TCA) and were then washed on the harvester using a 3% TCA solution. The washing was repeated several times in one case. Results of such an experiment are shown in Table 1. It is apparent that precipitation of cellular material is not necessary and saline wash provides the same values as after the TCA precipitation and washing.

Concentration of Cells and Phytoimitogens in Culture. The effect of cell concentration on lymphocyte transformation using flat-bottom microculture system has been described by Janossy and Greaves (1971). These authors have reported the optimal concentration of cells to be

4×10^5 per culture well, the transformation being proportional to the number of cells to this point but declining sharply if the number of cells exceeded this limit. Our system generally employed less than or equal to this number of cells.

The cell preparation described above generally yielded more than 95% viable lymphocytes. Because of this constant recovery it is possible to use an electronic counter (Coulter, Inc.) after lysing the red cells.

The optimal amounts of phytoimitogens, PHA and PWM are also described by Janossy and Greaves (1971). Excess of mitogen generally results in inhibition of lymphocyte transformation. The concentrations were optimized to provide the above mentioned values using the available mitogens. It is suggested that the concentrations be optimized for each preparation used.

Variability in Replicates and Within Animals. Variability within replicates of the same culture system is inherent in these procedures (Graf and Mather, 1972). In our system a 10% variation between different replicates was usual but occasionally larger variations were observed. Sufficiently large animal-to-animal variations were often noted. Graf and Mather (1972) have suggested a nonparametric analysis of variance using geometric means of replicates. In our experience, use of parametric test methods was sufficient to detect effects of chemical treatments. Use of a nonparametric system, although more

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sensitive, was not employed since we do not know the causes of variability and the related implication. Sensitive statistical tests for significance may, however, be applied if considered suitable.

Influence of Age on Lymphocyte Transformation. In many toxicologic studies, growing animals are used for short term repeated exposures to chemicals. Like certain other effects, the lymphocyte transformation is also altered with age. Adult animals possess considerably fewer lymphocytes that will form either spontaneous blasts or can be stimulated by phyto mitogenic agents. Fig. 3 and Table 2 illustrate the effects of age in different experiments. These experiments were not conducted separately but the data were obtained from control groups used in several other experiments involving evaluation of chemical immunotoxicity. It is apparent that there is a relatively rapid decline in spontaneous blast formation of mouse splenic lymphocytes and also of the stimulation indices obtained with PHA and PWM. In immunotoxicologic evaluation, the use of juvenile animals may have some advantage, although the variability is reduced considerably in older and mature animals. At the same time the immunotoxicological effect of chemical exposure is also considerably reduced in adult animals (Vos and Moore, 1974; Sharma et al., 1977b).

A drastic decline with age in one of the experiments shown in Fig. 3 may have been due to the stress produced by housing conditions. The animals in this group were housed in inhalation chambers and were

deprived of food and water during simulated exposure (although these were used as controls for respective exposure groups). Repeated handling of cages and other stress factors such as water deprivation for an extended period may be responsible for such a decline. Stress induced modulation of immune responses has been described previously (Monjan and Collector, 1977) where environmental stress both depressed and enhanced the immune responsiveness. In immunotoxicologic studies, an appropriate control, exposed to similar environmental conditions, is, therefore, highly desirable.

Addition of a Metabolizing System or Other Tissues to the Cultures. In the case where a chemical exposure alters the transformation of lymphocytes, addition of the chemical to the culture system directly in vitro can assess whether the effect is due to the chemical per se. It is possible that the chemical exerts its effect in vivo after being either itself modified chemically (biotransformation) or by modifying some tissue components (hapten conjugation). To verify such cases it was considered desirable that the chemical be added to culture simultaneously with an active metabolizing system or tissues like serum or liver from exposed animals. Addition of different amounts of serum and liver homogenate was thus attempted and the representative data are given in Table 3.

It appeared that even a small amount of these biomaterials, as little as 5 μ l of serum and 0.05 μ g of liver tissue to a 200 μ l culture system

produced appreciable inhibition of the lymphocyte transformation. A reduction in both spontaneous transformation as well as in mitogen induced transformation was observed. The effect of serum was surprising since the culture medium contained 10% fetal calf serum (heat-inactivated). Janossy and Greaves (1971) have suggested that fetal calf serum stimulates lymphocyte transformation. In another report, Vos and Moore (1974) reported that rat serum inhibits cell transformation in cultured thymocytes or splenic lymphocytes.

In a subsequent experiment, manipulations were made to serum and liver supernatant to determine the nature of inhibitory factors. The results of such a preliminary experiment are shown in Table 4. Heating (56°C for 1 hour) and dialyzing (in a dialysis bag, 15,000 daltons cut-off, against 0.01 M phosphate buffer, pH 7.4, for 18 hours) appeared to reduce the inhibitory effect of mouse serum only partially. Similar treatments were, however, not effective in the case of liver supernatant. When purified microsomes (100,000 g pellet from a post-mitochondrial, 9,000 g supernatant) were used in an amount equivalent to the added liver tissue, these were not found to be inhibitory. At this stage, the nature of inhibitory factor(s) in mouse serum and liver against its own splenic lymphocytes is not clear but some other reports also support this contention. In addition to the inhibitory effect of rat serum referred to above, serum and liver factors were reported to be inhibitory for hepatocyte multiplication in culture (Nadal et al., 1976). Suppression of lymphocyte stimulation in culture was also reported by uterine and placental extracts (Kouttab et al., 1976).

Based on preliminary results, it may be suggested that the inhibitory factors in liver are primarily in the soluble fraction and the chemicals can be metabolized by utilizing a purified microsomal system in lymphocyte cultures if necessary. This hypothesis, however, has not been experimentally tested at this point.

Effect of Antigen Challenge on Lymphocyte Transformation. If an immunosuppressive role of a chemical has to be evaluated, it may be necessary to challenge the exposed animals simultaneously to a known antigen and determine the specific immune responses. In some of our immunotoxicology experiments we attempted such a technique. Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin was found to be immunosuppressive (measured by skin reactivity to tuberculin and antibodies to tetanus toxoid in sensitized animals) but exposure to vinyl chloride did not show a similar effect. This correlated well with the mitogen stimulation indices in lymphocyte cultures from animals exposed to these chemicals. In these experiments, the animals were challenged with a mixture of tetanus toxoid and Freund's complete adjuvant and the effect of this tetanus antigen challenge in control groups of mice is shown in Table 5.

In both cases, antigen challenge increased the spontaneous blast formation of lymphocytes in culture and also increased the stimulation indices against PHA and PWM. The increase in spontaneous transformation was similar to those observed with exposure to chemicals in some cases and no additional interactions of the two (chemical exposure and antigen

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challenge) were observed. Known immunosuppressants like 2,3,7,8-tetrachlorodibenzo-p-dioxin caused a decrease in the stimulation indices even after antigen inoculations.

Although these results suggest that antigen challenge caused a stimulation of spontaneous and induced lymphocyte transformation in culture, these differ somewhat from some of the earlier reports. In one case where chickens were injected with killed Mycobacterium tuberculosis organisms, the peripheral lymphocytes from these animals showed a variable effect to PHA (Visco and Buening, 1977). Only one out of four birds showed enhancement of the stimulation index, while all 4 showed increased stimulation when purified protein derivative (PPD) was added to the culture. Challenge of chickens to Marek disease virus (MDV) decreased the stimulation indices by PHA, PWM and Con A (Lu and Lapen, 1974). Our results in mice challenged to a bacterial antigen suggest that the splenic lymphocytes are sensitized to the phytoimitogens. In addition to using an antigenic challenge for immuno-toxicologic evaluation, it is imperative that the health status of animals in such studies should be carefully monitored, since the presence of infection can alter the results.

Evaluation of Selected Chemicals. Using mouse splenic lymphocyte transformation as one of the methods for toxicologic evaluation, a number of chemicals have been tested. The details of these are reported elsewhere (Sharma et al., 1977 a, b, c). A summary of these observations

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is listed in Table 6. Some of these chemicals and their biotransformation products were used for direct addition to cultures in vitro and these results are presented in Table 7. It is interesting that both vinyl chloride monomer and styrene caused enhancement of spontaneous lymphocyte transformation when the animals were exposed to these chemicals in vivo. In vitro, on the other hand, these chemicals were inactive, but their metabolites caused a similar stimulation. This illustrates the role of biotransformation in immunologic effects.

CONCLUSIONS

Based on the testing of a limited number of chemicals, it may be said that splenic lymphocyte transformation *in vitro* can be considered as a model system for the evaluation of the effect of chemicals on immune mechanisms. Unless a good correlation by other testing methods is established, it should be used as a supplementary test in conjunction with other tests on the immune system, i.e., an effect on the immune responses to antigenic stimulation. The mouse appears to be a satisfactory species but age of the animal, housing and care, and adequate controls should be considered in any testing protocol.

Since the responsiveness of lymphocytes to transformation is greater in young animals as compared to adults, the animals should be used before achieving maturity. Mature animals can, of course, be used for comparison.

Exact implication of alteration of lymphocyte transformation is not known at present. The responsiveness of lymphocytes to phyto-mitogens is said to involve the same derepression phenomenon that is induced by antigenic substances in vivo (Oppenheim, 1968). In addition, phyto-mitogens cause a selective induction of DNA synthesis and this can be used as a tool for measuring the responsiveness of different populations of lymphocytes (Andersson et al., 1972; Janeway and Greaves, 1971; Stockman et al., 1971). Mouse splenic cells have relative selectivity

for some mitogens and in case of the two used in this report, mouse B-lymphocytes are stimulated by PWM, whereas T-cells exhibit selectivity to PHA (Janossey and Greaves, 1971; Stockman et al., 1971). PHA is considered as a general nonspecific stimulant in other species (Visco and Saening, 1977). Regardless of their selectivity it is desirable to use several mitogens. In a mixed cell system, such as the one described above, cooperation among different cells is likely and the activity of one may influence the transformation of cells in another subpopulation of lymphocytes.

Although it is desirable sometimes to use a purified population of lymphocytes, it is difficult for routine testing of chemicals. Manipulation of lymphocytes has been known to alter their transformation properties, e.g. lymphocyte activation by cell separation procedures has been reported (Wardley et al., 1976).

An alteration of stimulation indices to phyto-mitogens by exposure of animals to chemicals may be considered as an effect on immune mechanisms. Depression of such indices is comparable to immune suppression in the organism, as was the case with 2,3,7,8-tetrachlorodibenzo-p-dioxin. The exact implication of an increased stimulation index is not yet apparent but it may be considered as an immune enhancement or modulation of immune responses. Such a phenomenon has been described in the case of levamisole (Renoux et al., 1976).

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Influence of chemical exposure on spontaneous blast formation on lymphocyte cultures is difficult to explain. Several chemicals tested by this protocol exhibited this phenomenon, i.e. increased transformation in culture. Such an effect, however, should not be considered universal or nonspecific since exposure of male mice to toxic heavy metals did not produce such effect, although the stimulation indices to mitogens were altered (Gaworski and Sharma, 1977). Orpud and Cohen (1974) have implied that increased lymphocyte sensitivity, particularly that of T-cells is indicative of autoimmunologic effects. It would not be unlikely that such effect is produced by hapten-conjugation mechanisms and is similar to in vivo antigenic stimulation. If such is the case, the sensitizing potential of chemicals can be tested using these techniques. A direct evidence of this effect can be made if the sensitized lymphocytes show increased transformation in the presence of chemicals in culture, but often factors such as biotransformation and specificity towards a conjugating-macromolecule in the organism may make such testing difficult, if not impossible.

A reduction in lymphocyte transformation may be indicative of immune suppression and may be mediated either through damage of lymphatic organs (as appears to be the case in high doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin) or through steroid suppression mechanisms. The latter may be the reason for decreased responsiveness in adult animals or the animals exposed to an environmental stress.

In some of our studies, the enhancement of spontaneous lymphocyte transformation by chemical exposure was similar to one caused by antigenic stimulation, and the fact that these two did not have an additive effect, further supports the argument that this may be an autoimmunologic phenomenon.

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Table 1

EFFECT OF WASHING WITH TRICHLOROACETIC ACID (TCA)
ON SPLENIC LYMPHOCYTE CULTURES^a

Treatment	DPM/10 ⁶ cells	Stimulation Index	
		PHA	PWM
Wash with Saline	25673	4.0	6.9
Wash with TCA once	29476	3.3	5.2
Wash with Saline	30122	3.5	6.4
Wash with TCA repeatedly	31251	3.6	5.7

^a The cells were treated with and washed with a 3% solution of trichloroacetic acid to cause precipitation of nucleic acids and proteins. All cultures run in quadruplicate and averaged.

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Table 2

INFLUENCE OF AGE OF GROWING MICE ON THE MITOGEN INDUCED
STIMULATION INDICES OF SPLENIC LYMPHOCYTE TRANSFORMATION IN CULTURES

Experiment Number ^a	Approximate Age, days	Stimulation Index ^b	
		PHA	PKM
1	32	4.6±1.8	31.1±5.1
	46	2.8±0.4	12.4±1.2
	60	15.9±6.1	17.6±5.0
	88	1.7±0.2	5.1±0.4
2	43	4.6±1.7	10.1±1.9
	57	2.9±1.1	4.2±1.6
	71	2.3±0.7	2.2±0.4
	99	1.4±0.2	0.7±0.2
3	38	8.4±2.2	10.9±2.4
	43	3.6±0.8	6.8±1.0
	50	3.2±0.8	4.5±0.5
	64	4.2±0.7	4.1±0.8

^a The first two experiments are same as those also indicated in Fig. 3.

^b Mean±SE, 4 animals/group, each culture in triplicate.

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Table 3

EFFECT OF MOUSE SERUM AND LIVER SUPERNATANT ON
LYMPHOCYTE TRANSFORMATION IN VITRO^a

Agent and amount added,	DPM/10 ⁶ Cells ^b	Stimulation Index	
		PHA	PKM
None	8658±1174	3.8	4.4
Serum, 1 μ l	8077±2103	2.3	2.5
Serum, 5 μ l	5419±2052	3.1	2.1
Serum, 10 μ l	4710±1794	1.9	5.0
None	9252±3987	3.4	2.3
Liver, 0.25 μ l (0.05 μ g)	6039±1316	3.2	2.4
Liver, 2 μ l (0.4 μ g)	5794±1484	1.9	2.2
Liver, 10 μ l (2 μ g)	7613±3497	1.5	1.8

^a The Serum or 9000 g liver supernatant, 20% (post-mitochondrial fraction) was mixed with appropriate amount of media and added to culture wells. The equivalent amount of liver tissue is shown in parenthesis.

^b Mean±SE of 4 cultures.

Table 4

EFFECT OF DIFFERENT TREATMENTS ON MOUSE SERUM AND LIVER
SUPERNATANT ADDITION TO SPLENIC LYMPHOCYTE TRANSFORMATION
IN CULTURES^a

Agent and Treatment	DPM/10 ⁶ Cells	<u>Stimulation Index</u>	
		PHA	PSM
None	25190	4.0	6.6
Serum, untreated	1749	3.0	9.8
Heated Serum	5451	2.9	5.7
Dialyzed Serum	6643	2.1	3.6
Liver Supernatant, untreated	3129	2.7	1.2
Heated Liver Supernatant	1343	1.4	2.4
Dialyzed Liver Supernate	716	2.4	3.4
Microsomes from Liver	19590	1.7	3.7

^a The amount of different factors added to the cultures was, serum 10 μ l, liver 2.5 μ g of tissue equivalent, and microsomes obtained from liver reconstituted to represent 2.5 μ g of liver tissue. All values are average of 4 cultures.

Table 5
EFFECT OF ANTIGEN CHALLENGE ON SPLENIC LYMPHOCYTE
TRANSFORMATION IN CULTURES^a

Group	DPM/10 ⁶ Cells	Stimulation Index by	
		PHA	PMN
Control	6158±654	1.7±0.2	5.1±0.4
Antigen Treated	10764±2632	6.0±1.6	12.7±2.8
Control	2432±799	1.4±0.2	0.7±0.2
Antigen Treated	11736±3584	8.1±4.8	6.5±2.9

^a The animals were injected subcutaneously with 0.02 ml of an antigen mixture containing equal amounts of tetanus toxoid and Freund's complete adjuvant, 2 and 4 weeks before sacrifice and culture preparation. Mean and SE of 4 animals/group, all cultures conducted in triplicate.

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Table b

EVALUATION OF LYMPHOCYTE TRANSFORMATION TECHNIQUE
AFTER IN VIVO EXPOSURE OF MICE TO DIFFERENT CHEMICALS

Chemicals Evaluated	Spontaneous Transformation	Mitogen Induced Transformation
TCDD (2,3,7,8-tetrachloro-dibenzo-p-dioxin	Increased, not dose related, more pronounced after short time exposures	Decreased stimulation indices with both PHA and PWM, generally at high exposure levels (1 mg/kg/week or more)
Vinyl Chloride Monomer	Increase observed depending on exposure level and time, effect declining at later times	Increase seen both with PHA and PWM. The effect persisted throughout an eight week exposure period
Styrene	Dose and time independent increase observed. Effect not consistent	Little effect seen with both mitogens
Thiodiglycolic Acid	Slight increase, dose related	No consistent effect after two weeks of exposure

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Table 7

EVALUATION OF LYMPHOCYTE TRANSFORMATION TECHNIQUE AFTER IN VITRO
ADDITION OF CHEMICALS TO MOUSE SPLENIC LYMPHOCYTE CULTURES

<u>Chemicals Added</u>	<u>Spontaneous Transformation</u>	<u>Mitogen Induced Transformation Index</u>
2,3,7,8-tetrachlorodibenzo-p-dioxin	Dose related decrease	No effect
Vinyl chloride monomer	No effect	No effect
Thiodiglycolic acid ^a	Stimulation at 10^{-3} M	Slight increase
N-acetylhydroxyethyl cysteine ^a	No effect	No effect
Styrene	No effect	No effect
Styrene oxide ^b	Stimulation at 10^{-6} M and inhibition at 10^{-4} M or more	No effect

^a Reported metabolites of vinyl chloride monomer (VCM).

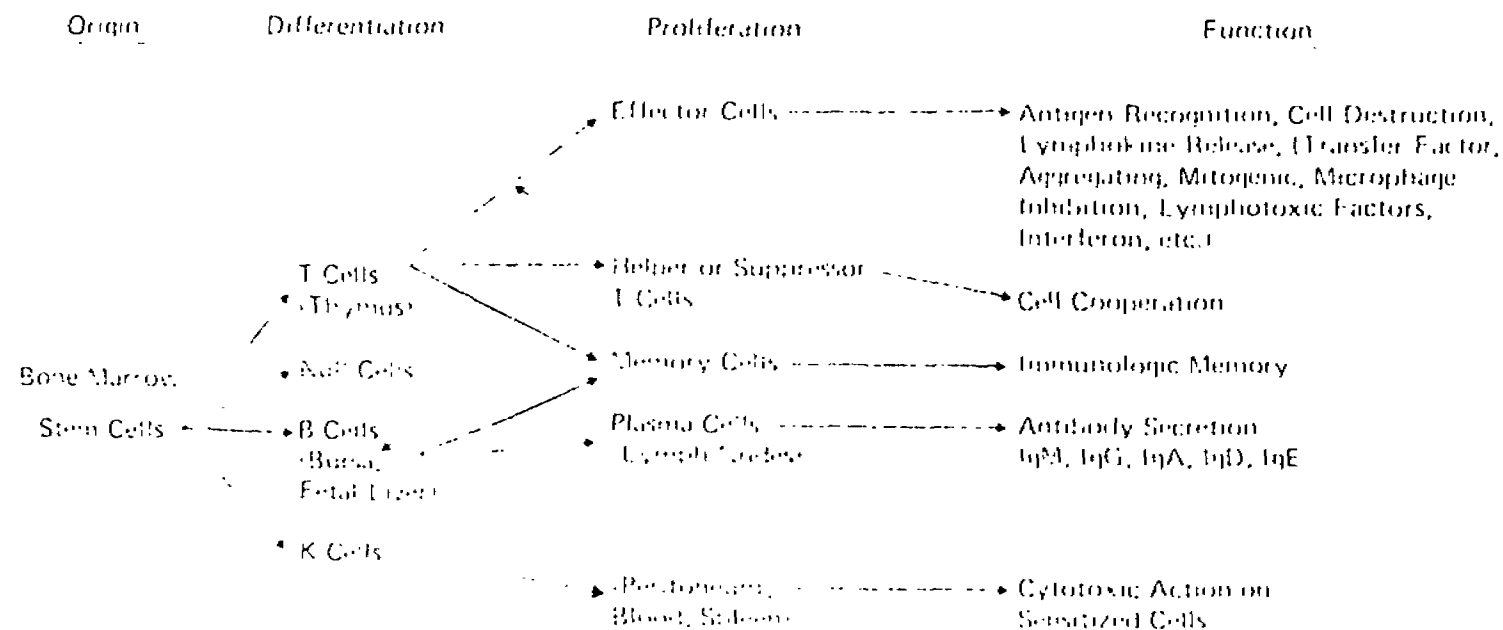
^b Transformation product of styrene in the body.

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LEGENDS TO FIGURES

- Fig. 1 Schematic representation of origin, differentiation and function of different populations of lymphocytes.
- Fig. 2 Suggested arrangements for arranging lymphocyte cultures in microplates: a, cultures run in triplicate; and b, cultures in quadruplicate. In each case a total of 8 samples can be used. The last two rows in "a" can be used for additional samples continuing in another microplate.
- Fig. 3. Thymidine uptake representing blast formation in lymphocyte cultures from mouse spleen as a function of age in growing animals. The data shown here has been taken from control groups of two independent experiments. In experiment shown by solid circles (●), the male animals were housed in groups of two each and given a dose of corn oil once a week. In the other one, with crosses (X), the males were housed in groups of four and were kept in inhalation chambers starting at the age of 32 days. Each point is an average of 4 animals, all cultures run in triplicate.

LYMPHOCYTE DIFFERENTIATION AND FUNCTION



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Figure 1

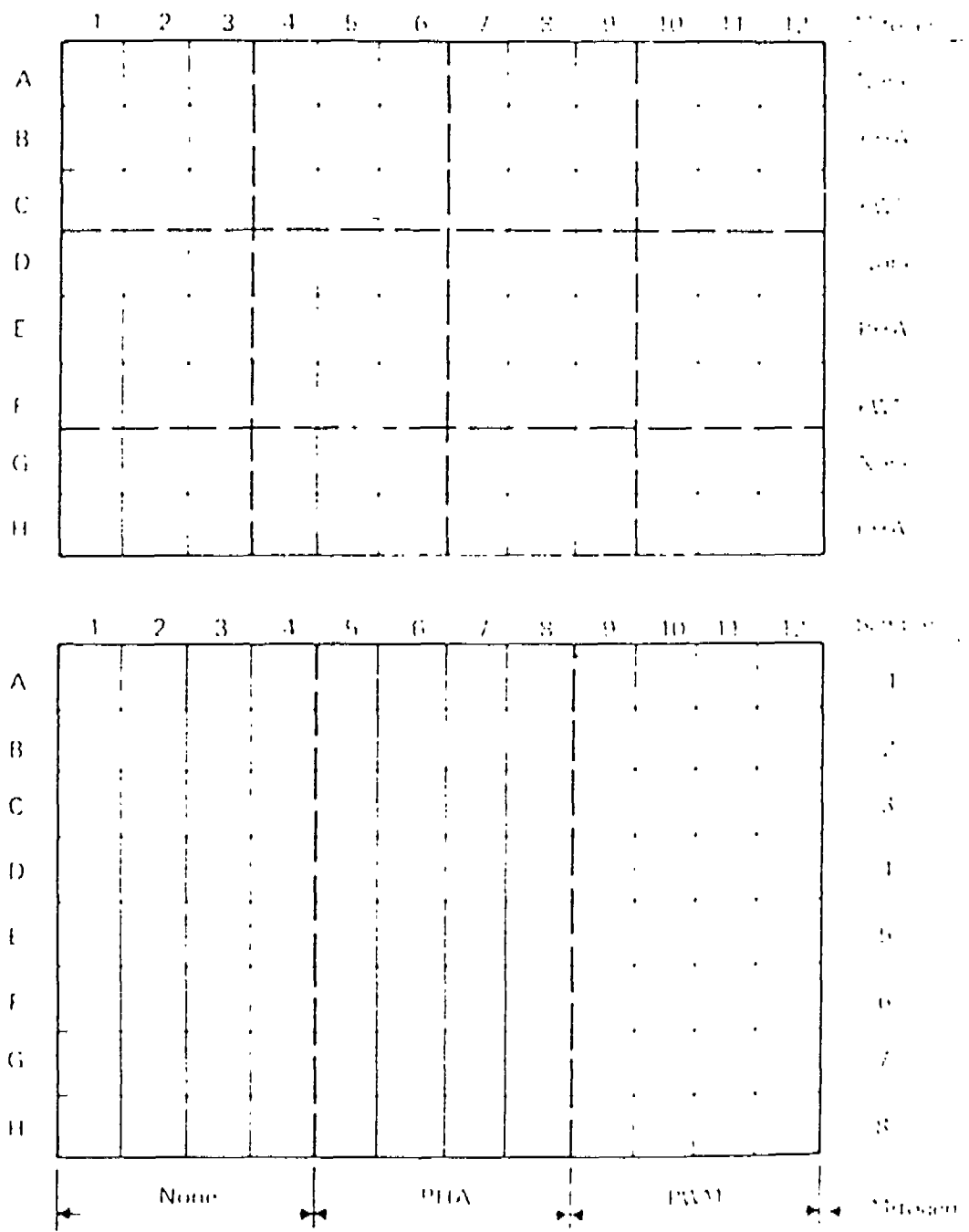


Figure 2

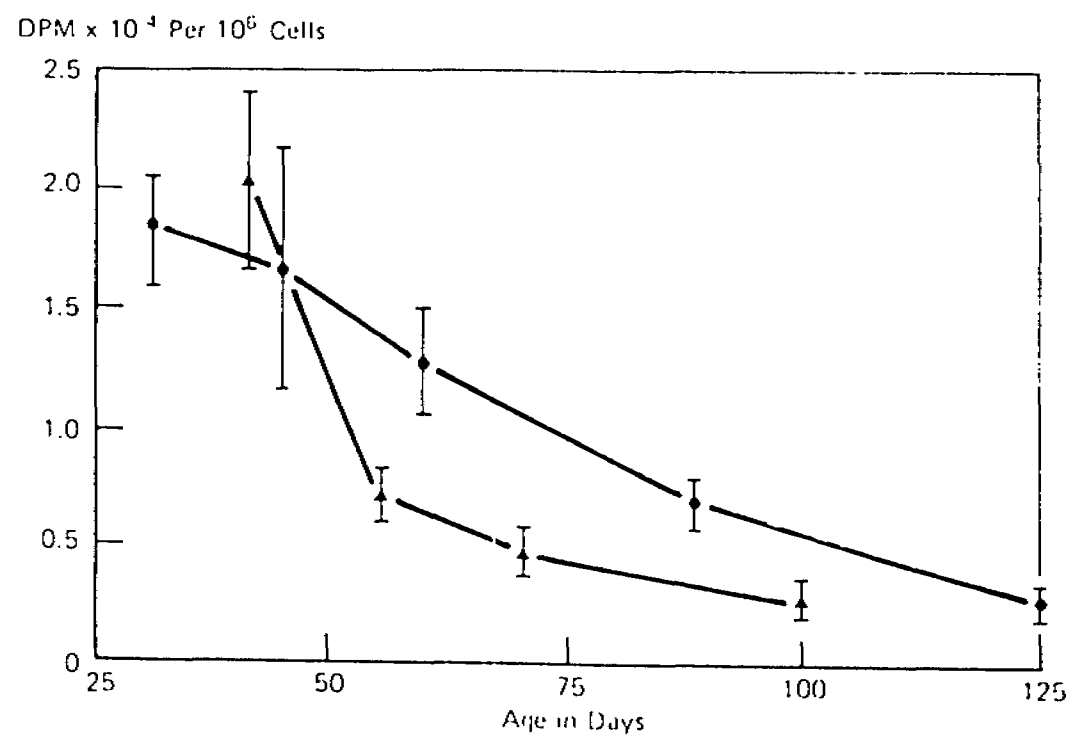


Figure 3

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