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Responses of Guinea-pig Lymphocytes to Mitogens, an Antigen, and Mixed Leucocyte Culture in Media with and without Mercaptoethanol and Foetal Calf Serum

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Summary. The ability of guinea-pig spleen and lymph node cells to undergo a proliferative response *in vitro* in the presence of mitogens (concanavalin A and lipopolysaccharide), a specific antigen (oxidized ferredoxin), and allogeneic cells was assessed under a variety of conditions. Time and dose dependency of the responses was measured in RPMI 1640, RPMI 1640 plus mercaptoethanol (ME), RPMI 1640 plus foetal calf serum (FCS), and RPMI 1640 with ME and FCS. Mitogen responses were also measured after treatment of the cells with sheep anti-guinea pig immunoglobulin (SaGPIg) and complement (C′) or after passage through nylon wool columns. Lipopolysaccharide (LPS) stimulated the cells under all media conditions over a wide range of concentrations but over a narrow time period. Nylon wool treatment of the cells eliminated the LPS response while SaGPIg and C′ reduced it. Concanavalin A (con A) stimulated the cells under all test conditions and demonstrated a dose–time interrelationship in terms of maximum response. Pre-treatment of cells with SaGPIg and C′ enhanced the response to con A while nylon wool fractionation diminished it somewhat. Only lymph node cells responded *in vitro* to oxidized ferredoxin (OFd). In serum-free media the OFd responses were maximal at 48 hours whereas in media containing FCS proliferative responses were supported for a prolonged period and appeared to be bimodal. Except for an early response with RPMI 1640 and ME, only media containing FCS supported stimulation in the mixed leucocyte culture.

INTRODUCTION

The *in vitro* stimulation of lymphocytes by mitogens, antigens or allogeneic lymphoid cells has been used as a correlate of cell-mediated immunity except in instances where B-lymphocyte mitogens were used. However, there is some question regarding what populations of cells are responding and whether or not macrophages are involved (Mugraby, Gery and Sulitzeanu, 1974). Various types of responses of lymphoid cells to a number of mitogens have been demonstrated in several animal species. In the mouse it is recognized that different lymphoid cell populations will respond to certain mitogens. Concanavalin A (con A) and lipopolysaccharide (LPS) have been shown to be specific mitogens for T and

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B lymphocytes, respectively (Anderson, Möller and Sjöberg, 1972b), although it has been demonstrated that con A will stimulate B lymphocytes if it is appropriately presented (Anderson, Edelman, Möller and Sjöberg, 1972a).

Murine T lymphocytes may be separated by passage of lymphocytes through nylon wool columns (Julius, Simpson and Herzenberg, 1973), or by treatment with anti-mouse immunoglobulin plus complement (C′) (Takahashi, Old, McIntire and Boyse, 1971). Conversely, populations of cells free of T lymphocytes may be prepared by treatment with anti-θ and C′ (Raff, 1969; Lamelin, Lisowska-Bernstein, Matter, Ryser and Vassalli, 1972). The guinea-pig system has not been so well elucidated. In the present study, treatment of cells with nylon wool or SaGPIg and C′ was examined to observe their effects on the con A and LPS responses.

Recently, reports have appeared demonstrating that lymphocytes can be cultured for relatively short periods of time without serum (Vischer, 1972; Coutinho, Möller, Andersson and Bullock, 1973). *In vitro* systems in which serum can be eliminated have several advantages; lower background controls, simpler characterization of supernatant factors, elimination of variability between batches of serum, and reduction of non-specific stimulation.

Several investigators have reported that the addition of reducing agents such as mercaptoethanol (ME) and cysteine has a beneficial effect in their culture systems. Greater viability and enhanced response to antigens, mitogens and mixed leucocyte cultures (MLC) have been claimed (Chen and Hirsch, 1972; Bevan, Epstein and Cohn, 1974; Broome and Jeng, 1973; Fanger, Hart, Wells and Nisonoff, 1970; Heber-Katz and Click, 1972).

This study was undertaken to elucidate some of the parameters governing the proliferative response of guinea-pig lymph node and spleen cells to various stimuli when cultured in media with and without FCS and/or ME, and to correlate some of the data presented here with information already available in murine systems.

MATERIALS AND METHODS

Animals

Outbred albino guinea-pigs of either sex weighing approximately 400 grams were used in all experiments.

Mitogens

Con A (Sigma, St Louis, Missouri) was made up in phosphate-buffered saline (PBS) to 200 μg/ml, sterilized, and stored frozen. LPS-W from *S. typhimurium* (Difco, Detroit, Michigan) was dissolved in PBS at 10 mg/ml, the pH adjusted to 8·0, heated for 30 minutes in a boiling water bath, and stored frozen. All further dilutions were made in serum-free medium.

Antigen preparation and immunization

Ferredoxin from *C. pasteurianum* (Sigma) was performic acid oxidized (Mitchell, Levy and Nitz, 1970) before use. Animals were injected in five places with 250 μg of oxidized ferredoxin (OFd) in 50 per cent Freund's complete adjuvant (FCA) using a total volume of 0·5 ml per animal. The animals were boosted similarly after 14 days and killed 10 days later. OFd used in cultures was dissolved in medium, sterilized, and stored frozen.

Preparation of cells

Spleens and lymph nodes were removed aseptically, teased into PBS, and the resulting cell suspensions transferred to plastic tubes. The cells were spun down (180 ***g*** for 5 minutes) and resuspended in 0·85 per cent NH_4Cl (in 0·01 M PO_4, pH 7·2) for 4–5 minutes to lyse the red blood cells. After three washes with PBS they were counted, using trypan blue to assess viability.

Media

The basic medium used for all experiments was RPMI 1640 (Gibco, Grand Island, New York) supplemented with 100 u/ml of penicillin, 100 μg/ml of streptomycin and 50 μg/ml of fungizone. ME was made up aseptically in medium at $2{\cdot}5 \times 10^{-4}$ M and stored frozen. Foetal calf serum (FCS) (Gibco, number 84557) was inactivated at 56° for 30 minutes and made up to 20 per cent in medium.

Cell culture

Spleen and lymph node cells were cultured in microculture plates. Mitogens or antigen in medium were added in a volume of 0·05 ml/well. When ME or FCS was used, 0·05 ml of the previously described stock solutions was added to each well. Five to 10×10^5 cells in 0·10 ml of medium were added to each well along with whatever volume of medium was required to make a final volume of 0·25 ml. This procedure resulted in a final concentration of ME at 5×10^{-5} M, FCS at 4 per cent, and cells between 2 and 4×10^6/ml. The MLC were set up in a similar manner except that $2{\cdot}5 \times 10^5$ cells in 0·05 ml from each animal were used per well. Control wells contained 5×10^5 unmixed cells. Background counts were obtained by averaging the control counts of each animal.

Labelling and harvesting

Tritiated thymidine (Amersham-Searle, Arlington Heights, Illinois) (specific activity 2·0 Ci/m mole) at a concentration of 1·0 μCi in 0·05 ml was added to the wells 18 hours before harvesting. Harvesting was performed by aspirating the contents of each well onto glass fibre filters using a multiple sample harvestor (Hartzman, Bach, Bach, Thurman and Sell, 1972; Thurman, Strong, Ahmed, Green, Sell, Hartzman and Bach, 1973). The filters were dried and counted in a scintillation counter.

Nylon wool treatment

Cells treated with NH_4Cl and washed as described above were suspended in PBS plus 5 per cent FCS and placed in plastic tissue culture plates for 1 hour at 37° to remove adherent cells. PBS with 5 per cent FCS was used for all subsequent nylon wool column manipulations. The non-adherent cells were washed into a nylon wool column, incubated for 1 hour at 37° and eluted slowly. The column consisted of a 25-ml syringe barrel filled to the 20-ml level with loosely packed nylon wool and sterilized. The procedure was adapted from that used by Julius *et al.* (1973). Recovery of cells was approximately 15 to 20 per cent of those applied. To obtain sufficient numbers of cells for some experiments, lymph node and spleen cells were pooled before filtration. These cells are referred to as pool cells.

Killing with anti-immunoglobulin plus complement

Ammonium chloride treated cells were taken up in sterile, inactivated sheep anti-guinea-pig immunoglobulin (SaGPIg) or guinea-pig C′ (each was diluted 1:4 in PBS) or

in both and incubated with intermittent mixing for 1 hour at 37°, after which the cells were washed and counted. 0·2 ml of the diluted SaGPIg and C′ were used per 10^7 cells. Where necessary, lymph node and spleen cells were pooled before treatment to ensure adequate numbers of cells.

RESULTS

INDUCTION OF DNA SYNTHESIS BY LPS

The results of dose–response and kinetic experiments using spleen cells in serum-free medium are presented in Fig. 1a. The optimal dose was found to be 4 μg/ml after 24 hours in culture. In cultures with FCS, concentrations of 16 and 64 μg/ml stimulated well after 24 hours (Fig. 1b). Stimulation indices (SI) between 2·2 and 3·1 were found with LPS-induced spleen cells in medium plus ME at LPS concentrations from 4–256 μg/ml after 24 hours in culture. LPS-stimulated lymph node cells as shown in Table 1. The dose–response to LPS was quite broad in all tests carried out. Pooled cells did not respond as well as spleen or lymph node cells alone (Table 1).

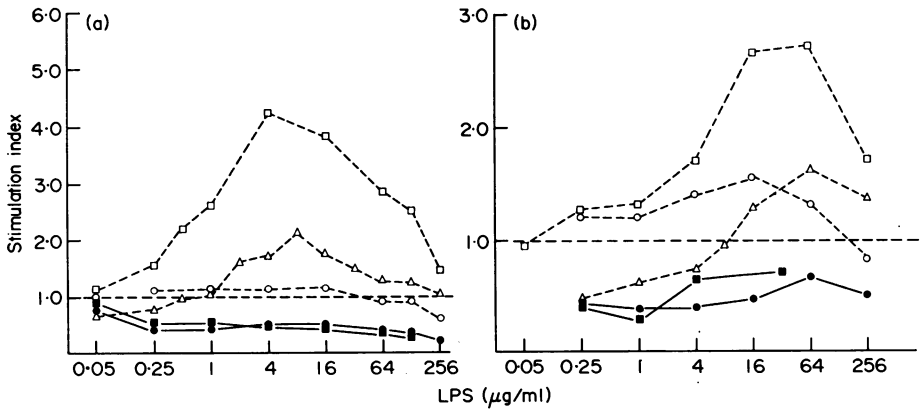

FIG. 1. The response of guinea-pig spleen cells to LPS in (a) serum-free medium and (b) medium with 4 per cent FCS. The cultures were labelled with tritiated thymidine for 18 hours after the following number of hours in culture: 0 (○ – – – ○); 24 (□ – – – – □); 48 (△ – – – – △); 72 (●——●); 96 (■——■).

INDUCTION OF DNA SYNTHESIS BY CON A

Similar experiments were done using con A. The results of spleen cell stimulation in serum-free medium are presented in Fig. 2a. The maximum response was found after 48 hours of culture using 1 μg/ml. Higher doses (4 and 8 μg/ml) induced moderate transformation after only 24 hours. The response with respect to the SI in medium with FCS was lower than the serum-free response (Fig. 2b). With FCS, peak stimulations were induced by 32 μg/ml at 24 hours and 1 μg/ml at 48 hours of culture. In con A experiments there was a trend toward high doses stimulating early and lower doses peaking at a later time, usually 48 or 72 hours. Lymph node cells produced much greater activation to con A than did spleen cells. Typical stimulations with 2 μg/ml of con A over 24, 48, and 72 hours were between 40- and 60-fold in all media tested. As observed with the LPS results, the highest SI were found using serum-free media.

EFFECT OF ANTI-IMMUNOGLOBULIN AND C′ OR NYLON WOOL ON THE RESPONSE TO LPS AND CON A

In most cases pre-treatment of the cells with SaGPIg and C′ enhanced the con A response and moderately reduced the response to LPS (Table 1). Only treatment of the cells with nylon wool consistently eliminated the LPS response; however, the con A response was also diminished somewhat (Table 1). Treatment with SaGPIg and C′ killed 23 per cent of spleen cells and 20 per cent of lymph node cells compared to controls containing normal sheep serum or C′ alone or SaGPIg alone.

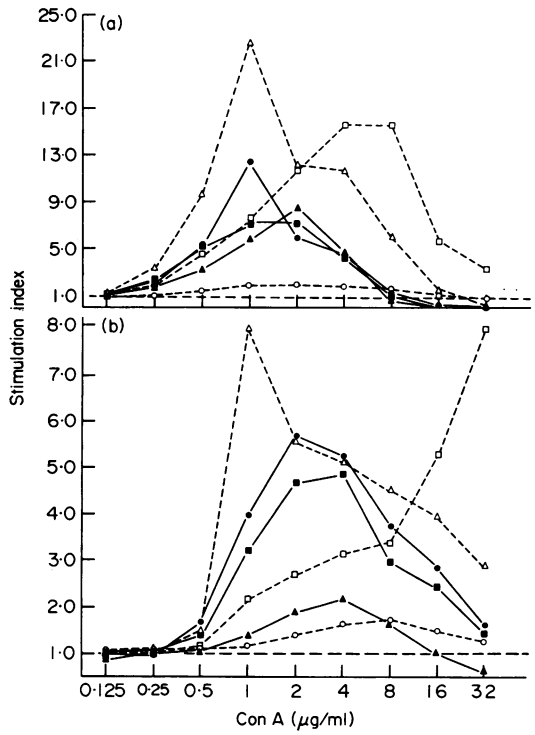

FIG. 2. The con A responses of guinea-pig spleen cells in (a) serum-free medium and (b) medium with 4 per cent FCS. An 18-hour labelling with tritiated thymidine was done after: 0 (○ – – – ○); 24 (□ – – – – □); 48 (△ – – – – △); 72 (●———●); 96 (■———■); 120 (▲———▲) hours in culture.

ANTIGEN-INDUCED LYMPHOCYTE TRANSFORMATION

The responses to OFd found in serum-free medium (Fig. 3a) and medium plus ME (Fig. 3b) were very similar. In both cases the response peaked at 48 hours with 32 μg/ml in medium only and 16 μg/ml in medium with ME. By 72 hours the SI had dropped sharply and by 96 and 120 hours was below unstimulated controls. The responses in medium plus FCS (Fig. 3c) and medium with FCS and ME (Fig. 3d) were bimodal. Both media supported good responses at 24 hours which declined at 48 and 72 hours and peaked again at 96 and 120 hours. The concentrations of OFd which induced the later response were often lower than those which stimulated the 24-hour response. Spleen cells did not

TABLE 1

EFFECT OF NYLON WOOL OR SaGPIg AND C′ TREATMENT ON THE CON A AND LPS RESPONSES OF GUINEA-PIG CELLS

Medium supplements	Source of cells	Hours of culture before labelling	Treatment	Con A (μg/ml)			LPS (μg/ml)					
				2	1	0·5	256	64	16	4	1	0·25
FCS	Spleen	24	None	—	—	—	1·68	2·71	2·64	1·69	1·31	1·25
			Nylon wool	—	—	—	0·76	0·52	0·79	0·80	0·79	0·92
FCS and ME	Spleen	24	None	10·5	3·50	1·10	—	—	—	—	—	—
			Anti-Ig and C′	9·10	5·02	1·22	—	—	—	—	—	—
FCS and ME	Node	24	None	42·9	33·2	3·99	—	3·42	3·36	3·44	—	—
			Anti-Ig and C′	70·7	50·8	11·9	—	2·20	2·30	1·94	—	—
FCS and ME	Pool	24	None	27·4	—	—	—	—	2·17	1·86	—	—
			Nylon wool	14·6	—	—	—	—	0·75	1·03	—	—
			Anti-Ig and C′	31·1	—	—	—	—	1·75	—	—	—
FCS and ME	Pool	48	None	—	—	—	—	—	1·06	0·73	—	—
			Nylon wool	—	—	—	—	—	0·70	0·45	—	—

The results are expressed as stimulation indices.

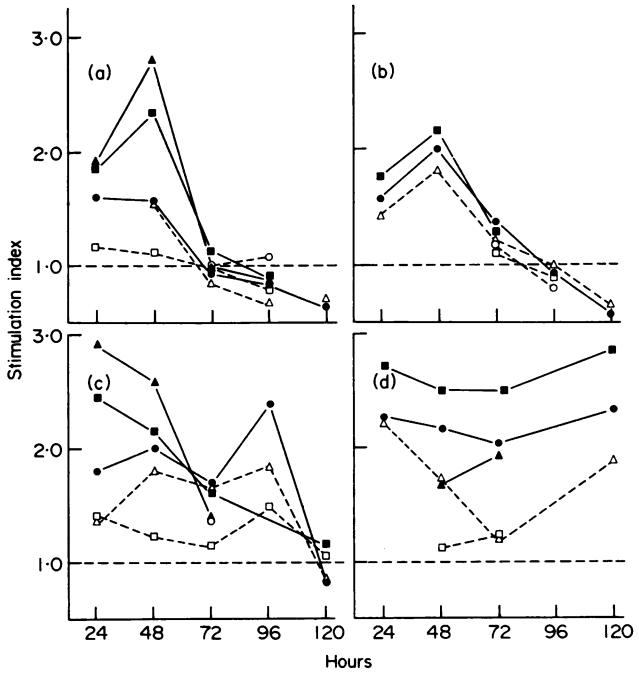

FIG. 3. The response of OFd-immune guinea-pig lymph node cells to OFd at: 1 (○ – – – ○); 2 (□ – – – – □); 4 (△ – – – – △); 8 (●———●); 16 (■———■); 32 (▲———▲) μg/ml. The cultures were labelled for 18 hours beginning at the times indicated on the figures. The media used were: (a) medium only; (b) medium with ME; (c) medium with FCS; (d) medium with FCS and ME.

respond under any of the conditions used even though lymph node cells from the same animal were able to respond. Lymph node and spleen cells from control animals immunized with FCA only were not stimulated by OFd.

MLC RESPONSE OF LYMPH NODE AND SPLEEN CELLS

Data from the MLC tests has been summarized in Table 2. Medium alone was unable to support a response at any time during the culture period. Except in the case of lymph node cells at 96 hours, very little stimulation was found in medium with ME. Media with

TABLE 2

MLC TESTS OF ALLOGENEIC GUINEA-PIG LYMPH NODE AND SPLEEN CELLS IN FCS- AND ME-SUPPLEMENTED MEDIA

Medium only		Medium plus ME		Medium plus FCS		Medium plus ME and FCS		Day†
Nodes*	Spleens	Nodes	Spleens	Nodes	Spleens	Nodes	Spleens	
1·38	1·08	1·33	1·08	1·56	1·22	1·36	1·16	2
0·93	0·92	2·00	0·80	1·34	1·35	2·05	1·45	4
0·89	1·20	1·13	1·22	2·13	2·29	2·54	3·35	6
0·96	1·20	0·76	1·28	1·53	0·82	1·52	3·87	8
1·22	1·34	0·76	1·44	1·28	0·61	0·56	2·09	10

The results are expressed as stimulation indices.

* Source of cells.

† The cultures were labelled for 18 hours after incubation for the indicated length of time.

FCS or FCS and ME supported normal responses, however, the response with FCS and ME was better than in medium with FCS only.

DISCUSSION

The results presented here have shown that guinea-pig lymphocytes can be readily cultured and stimulated by mitogens and an antigen (OFd) in serum-free medium. With the addition of ME and/or FCS to the medium, a mixed lymphocyte reaction was also detected.

Media composition had a marked effect on the control background counts in the stimulations. Average background counts when cultures were tritiated at 24 hours were 899 (medium only), 2210 (medium plus ME), 1825 (medium and FCS), and 7252 (medium with FCS and ME). Although actual counts in mitogen stimulations were higher in media containing ME and/or FCS, the stimulation indices were, on the average, higher in media without serum and ME because the unstimulated controls were lower. The SI of OFd cultures were similar regardless of the type of media used, while the MLC were consistently better when the medium was supplemented with FCS and ME.

A wide range of LPS concentrations (0·25–256 μg/ml) was able to induce DNA synthesis and in the presence of ME there was little difference in the response of cells to 4–256 μg/ml LPS. The kinetics of the LPS response were unusual in that, regardless of the media used, significant stimulation was achieved only when the cultures were tritiated at 24 hours. At 0 and 48 hours only slight stimulation was found and by 72 and 96 hours the counts were below the unstimulated controls. The addition of FCS or ME shifted the dose response to slightly higher LPS concentrations.

Both the dose–response and kinetics of the con A stimulations were moderately altered by the use of FCS. Except for con A at 1 μg/ml, cultures containing FCS usually required higher concentrations of con A and the response was more prolonged. Other workers have noted that higher concentrations of con A are required in media with FCS and attribute this to the ability of con A to bind serum proteins (Möller, Andersson, Pohlit and Sjöberg, 1973).

Con A-induced DNA synthesis was substantially higher with respect to total counts and SI with lymph node cells than with spleen cells. This observation is consistent with the generally accepted view that there are greater relative numbers of T lymphocytes in lymph nodes than in spleen. It has been proposed that con A is a T lymphocyte-specific mitogen in chickens (Weber, 1973), guinea-pigs and rabbits (Elfenbein, Harrison and Green, 1973), and mice (Elfenbein *et al.*, 1973; Andersson *et al.*, 1972b). The results of our experiments using nylon wool and anti-immunoglobulin with C′ are in agreement with studies in other animal systems which indicate that LPS is a B-lymphocyte mitogen. Using different techniques, Elfenbein *et al.* (1973) have suggested that LPS is B lymphocyte-specific in guinea-pigs. Using anti-light chain and C′, Gmelig Meyling, Kooy-Blok, and Ballieux (1974) were able to kill 12 per cent of peripheral blood lymphocytes and 42 per cent of lymphocytes from human tonsils. Takahashi *et al.* (1971) using anti-light chain and C′ in mice were able to kill up to 50 per cent of spleen lymphocytes and 40 per cent of lymph node lymphocytes. Our results of 23 per cent killing of spleen cells and 20 per cent killing of lymph node cells are consistent with these findings, since spleen and lymph node cells rather than enriched populations of lymphocytes were used. The ability of nylon wool columns to remove cells bearing surface immunoglobulin (presumably B cells) has been

demonstrated by Julius *et al.* (1973). In our experiments, nylon wool treatment of guinea-pig cells completely eliminated the response to LPS, whereas treatment with anti-immunoglobulin and C′ only reduced it. Using rabbit-anti mouse immunoglobulin and C′ we have been able to reduce the LPS response of mice by more than 80 per cent (unpublished observations). That the guinea-pig LPS response is relatively insensitive to SaGPIg and C′ treatment could be due to an LPS-responsive population of cells with a low density of surface immunoglobulin. Nylon wool treatment also reduced the con A response. As nylon wool operates on a principle of differential adherence, it is possible that some of the cells capable of responding to con A are removed. Usually, the con A response was enhanced by treatment with anti-immunoglobulin and C′.

OFd induced thymidine uptake in lymph node cells only, but in all the media tested. Vischer (1972) using mice and Kirchner and Oppenheim (1972) with chickens have reported serum-free antigen stimulation using keyhole limpet haemocyanin and sheep red blood cells, respectively, as antigens. The kinetics of the serum-free OFd responses with and without ME were similar to each other and distinctly different from the kinetics of medium with FCS and medium with FCS and ME. It was unexpected to see the antigen stimulation in FCS and FCS plus ME containing media occur as early as 24 hours, decline at 72 hours (a time often considered to be optimal), and increase again at 96 and 120 hours. These results may indicate that two different cell populations were responding to the antigen at different times. The bimodal response was not observed in serum-free media, probably because without serum, cell viability was not adequate to support a second response. Preliminary experiments, not presented here, testing the response of anti-immunoglobulin and C′ or nylon wool-treated cells to OFd have been inconclusive, but suggest that SaGPIg and C′ treated cells may be able to respond, whereas nylon wool-treated cells seem incapable of responding. Mugraby *et al.* (1974) using mice demonstrated that depletion of T or B lymphocytes reduced the response to sheep red blood cells.

The use of ME had its most prominent effect in the MLC tests. The response in medium plus FCS and ME was greater and more prolonged than in medium with FCS only. No response was found in serum-free medium, but with the addition of ME, a detectable response was obtained. Bevan *et al.* (1974) using mouse spleen cells showed that addition of ME to serum free MLC tests resulted in positive responses. Similar results were obtained by Heber-Katz and Click (1972). Although the mechanism of action of ME and related compounds is unknown at this time, it has been observed that the presence of ME, cysteine, etc. greatly increases the viability of cells in culture (Chen and Hirsch, 1972; Heber-Katz and Click, 1972). Chen and Hirsch (1972) have used ME as a substitute for macrophages which also appear to have a beneficial effect on viability. It has been proposed that ME acts as a substitute for a macrophage-produced factor (Broome and Jeng, 1973).

It would appear that those responses which require prolonged culturing of lymphocytes cannot be supported in media lacking FCS, as noted in the late stimulation by OFd, or ME, as seen in the MLC tests. However, responses which are maximal within the early days of culture are adequately supported in the absence of these media components. The data reported here indicate that there are no major differences between the responses of guinea-pig and mouse lymphocytes in the mitogen, antigen and MLC test systems.

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