TABLE I. Effect of FUDR on Interferon Production by CE Cells Infected with Chickungunya Virus.

FUDR, molarity	Interferon titer		Virus titer $\log_{10}$ pfu/ml	
	No FUDR	With FUDR	No FUDR	With FUDR
3×10^{-6}	300	200	3.2	3.2
1×10^{-6}	100	100	3.4	4.0
3×10^{-5}	100	100	Not done	

FUDR, at indicated final concentrations, was added to the cells at the same time as the virus. Virus:cell multiplicity = 10. Virus was absorbed 30 min at room temperature, and cells were then washed 4 × with BME before incubation for 18 hr at 37°C. Interferon titers were determined on the fluid and virus on the frozen and thawed cells plus fluid.

TABLE II. Effect of FUDR on Growth of Vaccinia Virus in CE Cells and Production of Interferon by Such Cells.

FUDR	Interferon titer	Final titer of virus $\log_{10}$ pfu/ml
0	5	6.9
1.8×10^{-5}	10	5.5
1.2×10^{-5}	10	5.5
6×10^{-6}	8	5.8

Virus was added at a multiplicity of 8, at same time as FUDR. After 2 hr absorption at 37°, the fluid was removed, the cells were washed 3 times with BME, and new fluid with or without FUDR was added. After 24 hr at 37°, the whole culture was frozen and thawed 3 times and clarified by low speed centrifugation. Virus and interferon contents were determined in the supernatant fluid.

the production of interferon by these cells when infected with chickungunya virus, an RNA virus. Our strain of this virus grows little or not at all in CE cells, but gives maximum titers of interferon within 5-6 hours after infection.

Table II shows the effect of FUDR on the growth of vaccinia virus, a DNA virus, in CE cells and the production of interferon by these cells when infected with vaccinia virus.

It will be seen that vaccinia growth was inhibited about 95%, but that there was no inhibition of interferon production. The small differences in interferon titer seen in Table I are within experimental error.

Discussion and summary. FUDR had no effect on the production of interferon by CE cells, when the inducing virus was chickungunya virus, an RNA virus, or vaccinia virus, a DNA virus. The concentrations of FUDR used inhibited total DNA synthesis and growth of vaccinia virus by about 95%. Thus, under our experimental conditions, it may reasonably be concluded that little or no new DNA synthesis is needed for the production of interferon. Holmes *et al*(1), working with mumps virus, report that iododeoxyuridine inhibits the production of interferon without any gross toxicity to the cells. They conclude that new DNA synthesis is needed for interferon production. It is difficult to reconcile their findings with ours, unless the rather high levels of IUDR they used exerted a deleterious action on the metabolism of the cells which was not revealed by morphological alterations. The data of Donikian and Stewart(2) do not suggest the need for new DNA synthesis for the production of interferon. It would appear, therefore, that DNA is functioning in its usual way as a template in the interferon producing cell.

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Effects of Endotoxins on Cultured Leukocytes. (30033)

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In vivo administration of endotoxin produces multiple hematologic, immunologic, and physiologic effects(1). It can result in an immediate granulocytopenia, followed by lymphopenia and then a transient granulocytosis. Repeated administration results in

tolerance to the pyrogenic effects(2), progressive peripheral granulocytopenia(3), increased resistance to infection by Gram negative bacteria(4), enhanced host reticuloendothelial system (RES) phagocytosis(5), RE cell, macrophage, and lymphocytic hyperplasia(6), and transient elevation of many specific "natural" (bactericidal) antibodies thought to be due to a temporary increase of endotoxin stimulated cell production or release of preformed antibodies(7).

Endotoxin has been reported to enhance the primary antibody response to antigens in many animals. In spleen cell cultures obtained from rabbits stimulated simultaneously with bovine gamma globulin (BGG) and endotoxin, plasma cells appeared earlier and in greater numbers than in animals given BGG alone(8). Addition of endotoxins to cell suspensions has been reported to damage spleen macrophages(9) and cause degenerative changes of platelets and polymorphonuclear leukocytes from rabbit blood(10).

However, addition of 5 μmg per ml *Shigella shiga* and *Salmonella enteritides* endotoxins to *in vitro* cultures of peripheral white blood cells (WBC) failed to affect the lymphocytes(11).

The above reports suggested that further studies of the *in vitro* effects of various endotoxins on human leukocytes would be of interest. Their effect on thymidine H^3 uptake, mitotic index and leukocyte survival were investigated. It was found that the endotoxins stimulated the proliferation of small lymphocytes and were compatible with the selective survival of monocytes in short-term cultures of peripheral WBC. Ability of an endotoxin to stimulate lymphocytes in general was proportional to the degree of its *in vivo* antigenicity.

Experimental. Three healthy young adults were the source of the peripheral WBC used in the *in vitro* cultures. They had a history of the usual childhood immunizations, and 2 of them (A.B. and B.S.) also had received a typhoid vaccination 5 and 7 years respectively prior to the study.

Fifty ml of heparinized blood (10 μ/ml) were obtained by venipuncture and the red blood cells allowed to sediment at 37° for 1-2 hours at a 15-20° angle. The WBC rich plasma was withdrawn and added to a minimal essential medium* (supplemented with 50 U penicillin, 50 U streptomycin, and 0.1 mM glutamine/ml). The final leukocyte concentration of this cell suspension was 1000-2000/mm^3. Autologous cell-free plasma was added to all the cultures, and in the cell suspensions of A.B. and K.S., 10% calf serum† was also used to obtain a final total plasma concentration of 25-30%. The cell suspensions were divided into aliquots of 12 cc each. To one of the aliquots only, 0.5 ml isotonic saline was added. To the others, 5 types of bacterial endotoxins of varying dilutions in saline were added. The following endotoxins were studied: *Salmonella typhosa* 0901,‡ highly purified *Salmonella enteritides* (kindly supplied by Dr. Edgar Ribi), *Escherichia coli* 0127B8,‡ *Salmonella abortus equi* (Pyrexal),§ and Pseudomonas polysaccharide complex (Piromen).‖ The concentrations of endotoxin used are indicated in Table I.

The cultures were incubated for 5 days at 36.5-37.5°C, then harvested by centrifugation at 500 rpm (International centrifuge with No. 240 head) for 5 minutes. The supernatant was discarded and the cells were fixed for 10 minutes in modified Carnoy's solution (3:1 absolute ethyl alcohol to glacial acetic acid). The fixed cells were recentrifuged and resuspended in a small amount of fixative. They were then pipetted onto slides, air dried, and stained with Giemsa (Harleco).

The slides were coded and independently analyzed by 2 individuals for mitoses, monocytes, "transformed" lymphoblasts, and small lymphocytes. The mitotic index was defined as the number of mitoses per 1000 mononuclear cells. Unrecognizable cells and polymorphonuclear leukocytes were excluded. Small lymphocytes were easily identifiable by the usual criteria of size, scanty cytoplasm, and heterochromatic nuclei. The larger mono-

* Eagles Modified Spinner medium, Flow Labs, Rockville, Md.

† Calf serum from Microbiological Associates, Bethesda, Md.

‡ Difco Laboratories, Detroit, Mich.

§ Dorsey Laboratories, Lincoln, Neb.

‖ Travenol Laboratories, Inc., Morton Grove, Ill.

TABLE I. Comparison of Median Response of Peripheral WBC with Various Endotoxins to Unstimulated Culture's Response.

Type and conc of endotoxin	No. of exp	Subjects	Mitotic index	% Monocytes	% Transformed lymphocytes	% Labelled cells
S. typhosa .5 μμg–50 μg/ml	13	B.S.	2 (0–12)*	22 (17 –25)	7 (3 –29)	—
		K.S.	0 (0– 0)	26.5 (26 –27)	4.2 (4.1– 4.2)	3.9 (3.5– 4.3)
		A.B.	1.5 (1– 2)	33 (32 –34)	6 (5.5– 6.5)	5.4 (5.2– 5.5)
		Median	1.5	26.5	6	4.8
S. enteritides 50 μμg–10 μg/ml	7	B.S.	1 (0– 2)	25 (22 –28)	2.7 (1.3– 4.0)	—
		K.S.	1.5 (1– 2)	28 (25 –31.5)	15 (10 –20)	13 (9 –18)
		A.B.	2 (1– 3)	30 (22.5–37.5)	17 (10 –24)	11 (7.3–14)
		Median	2	26.5	10.3	11.8
E. coli 50 μμg–50 μg/ml	5	B.S.	2	49.0	2.4	—
		K.S.	.5 (0– 1)	16 (2.5–29)	15 (7.5–23)	11 (7.8–13.5)
		A.B.	2.5 (1– 4)	26 (18 –34.5)	13 (7 –19.5)	12.4 (6.3–18.5)
		Median	1	29.0	7.5	10.1
Piromen 50 μμg–3.3 μg/ml	3	B.S.	1	16.4	2.8	—
		K.S.	0	8.0	0	—
		A.B.	0	2.0	0	—
		Median	0	8.0	0	—
Pyrexal .05 μμg–50 μμg/ml	7	B.S.	0	0 (0 –35.4)	0 (0 – .4)	—
		K.S.	0	8.0	0	—
		A.B.	0	4.0	0	—
		Median	0	4.0	0	—
Unstimulated control	5	B.S.	0	22 (6.0–24.8)	.5 (.2– 1.2)	—
		K.S.	0	8.0	3.5	3.3
		A.B.	0	8.0	0	0
		Median	0	8.0	.5	1.7

* Numbers in parentheses indicate range.

cytes often contained phagocytized intracytoplasmic debris, and eosinophilic granules. The cytoplasmic membrane was often ill-defined, and the nucleus irregular in shape. The "transformed lymphoblast" usually had clearly delineated cytoplasmic membrane, basophilic nongranular cytoplasm, a rounded or oval shaped nucleus with prominent nucleoli, and was never seen to be phagocytic (12). However, occasional large mononuclear cells were difficult to classify because they contained features of both monocytes and transformed lymphocytes, or because their cytoplasm was absent.

Tritiated thymidine¶ (2 μc/ml) were added to the cultures of A.B. and K.S. 4 hours prior to harvesting. Radioautographs were prepared in the usual manner(13) and the proportions and types of labelled cells determined.

The patients' sera were tested for the presence of antibodies to the Somatic O antigens of Gram negative bacteria (Table III) using the heated whole bacterial cell agglutination technique(14). The reactions of known hyperimmune sera served as a control (Table III).

The antigenicity of the endotoxins was confirmed by the flocculation of endotoxin coated bentonite particles using the subjects and known hyperimmune sera(15) (Table III).

Results. The unstimulated control cultures after 5 days of incubation lacked mitoses (<1/2,000), contained 6-8% monocytes, and 0.5% (0-3.5%) transformed lymphocytes (Table I). Granulocytes, both viable and degenerated, ranging from a few to as high as 25% were present.

The addition of *S. typhosa* endotoxin was associated with a mitotic index of 1.5 (0-12), an increase in the proportions of monocytes to 26.5 (22-33%) and the appearance of 6% (4.1-7%) transformed lymphocytes. Similar results were obtained in cultures stimulated with *S. enteritides,* and *E. coli* endotoxins (Table I).

¶ $TdRH^3$ sp. act. 0.360 c/mM, Schwarz BioResearch Inc., Orangeburg, N. Y.

TABLE II. *In vitro* Lymphocyte Response of Subject (B.S.) to Identical Concentrations of Endotoxins at Various Times.

Date of exp	1000 Mononuclear cell differential	Unstimulated culture	*S. typhosa* 50–70 μμg/cc	*S. enteritides* 50–70 μμg/cc	Pyrexal 50 μμg/cc
2/ 7/64	Mitotic Index	0	2	—	—
	% Monocytes	6.0	17.8	—	—
	% Transformed lymphocytes	.5	5.0	—	—
2/27/64	Mitotic Index	0	2	2	Total cell
	% Monocytes	24.8	18.5	21.5	death
	% Transformed lymphocytes	1.2	3.0	4.0	
3/20/64	Mitotic Index	0	1	0	*Idem*
	% Monocytes	22.0	27.0	28.0	
	% Transformed lymphocytes	.2	5.6	1.3	

TABLE III. Comparison of Antibody Titers of Subjects and Hyperimmune Sera.

Techniques	Antigens	Subjects' sera B.S.	K.S.	A.B.	Hyperimmune sera
Bacterial agglutination	Salmonella B	16*	32	32	5120
	" D	8	32	16	5120
	E. coli 0127	512	32	0	20480
Bentonite flocculation	*E. coli*	16	2	1	2040†
	S. enteritides	4	1	4	8000†
	S. typhosa	4	0	0	32000†

* Reciprocal of titer. † See Ref. 15.

In contrast, Piromen resulted in minimal lymphocyte transformation of 2.8% and a mitotic index of 1 on only one occasion at the lowest concentration of 50 μμg/ml. At higher concentrations considerable cell death was evident, and only a few monocytes and damaged small lymphocytes survived. Pyrexal at concentrations above 0.005 μμg/ml caused total cell death, and at this lower concentration no lymphocyte transformation response was ever seen (Table I). There were generally fewer degenerate and viable granulocytes evident in the endotoxin treated cultures than in the unstimulated cultures.

The tritiated thymidine radioautographs revealed that 60-100% of the transformed lymphocytes and mitoses were labelled. No labelled small lymphocytes or monocytes were seen. In the cultures of K.S. and A.B., the proportions of cells labelled with H^3TdR correlated well with the proportions of transformed lymphocytes detected using only morphological criteria (Table I).

The lymphocyte transformation of these 2 subjects to *S. enteritides* and *E. coli* endotoxins was consistently greater than that of subject B.S. The unstimulated culture of K.S. also contained more transformed lymphocytes than expected. These findings were probably due to lymphocytic stimulation by the heterologous antigens present in the calf serum. This alone, in our experience, can result in 0.5-3.0% lymphocyte transformation in 5 days of culturing.

The leukocytes of subject B.S. were retested with identical quantities of endotoxin on 3 occasions. A moderate degree of variability of the lymphocyte transformation response to the endotoxins and of the unstimulated cultures' findings was noted (Table II).

All the subjects had low titers of circulating antibodies against the antigens of *S. typhosa* and *S. enteritides* of the Salmonella D group, *S. abortus equi* of Group B, and *E. coli*. Their sera agglutinated heated whole bacteria which is indicative of the presence of antibodies to the heat stable O antigens. The antigenicity of the *S. typhosa, S. enteritides* and *E. coli* was attested to by the flocculation of endotoxin coated bentonite particles using hyperimmune as well as the subjects' sera (Table III).

Discussion. The endotoxins derived from *S. typhosa, S. enteritides,* and *E. coli* when added to short-term peripheral WBC cul-

tures transformed some of the small lymphocytes to "lymphoblasts." These transformed cells were able to proliferate as indicated by their uptake of TdRH[3] and the presence of occasional mitoses. This response could be elicited over a wide range of endotoxin concentrations, which would probably prove lethal if administered *in vivo*. We are unable to correlate the proportions of lymphocytes that transformed with the concentrations of endotoxin that were used. The response was evident at concentrations as low at 50 $\mu\mu$g/ml of endotoxin indicating that the lymphocyte transformation response is a very sensitive means of somatic antigen detection.

It has been demonstrated that in short-term tissue cultures the majority of lymphocytes will in the presence of phytohemagglutinin (PHA)(16), staphylococcal filtrate (11), and streptolysin S(17), transform to large "blast-like" cells with a high mitotic index. It also has been demonstrated that lymphocytes obtained from a tuberculin sensitive donor when stimulated with PPD *in vitro* will transform to "blast-like" cells and undergo mitosis(18). This response has since been reported with many different antigens provided the cell donor has had a previous contact with the substance(19). Antigens usually transform less than a third of the lymphocytes in 5 days of incubation, and at a slower rate than does PHA(20). Thus, the lymphocyte response to endotoxins *in vitro* is similar to their response to antigens. Endotoxins presumably transformed those lymphocytes that have had previous exposure to these antigens of Gram-negative organisms which are also present in the endotoxins. The demonstration of circulating antibodies in the subjects is evidence of their previous exposure to these polysaccharide antigens. That the particular endotoxins utilized were antigenic was confirmed by the bentonite flocculation technique. The particular endotoxin preparations employed in this study, when administered *in vivo,* have resulted in a rise in the antibody titers to somatic O antigens (15). Recent studies done in collaboration with Dr. Sheldon Wolff** indicate that different lots of an endotoxin may vary in their ability to produce cytotoxicity and lymphocyte transformation. Furthermore, 4 subjects immunized with *S. typhi* endotoxin have failed to manifest a significant increase in *in vitro* lymphocyte transformation. If anything, the cytotoxic effects of the endotoxins on their cultured leukocytes increased with the rise in antibody titers.

The potential of a particular endotoxin to transform lymphocytes *in vitro* correlates with its capacity to stimulate antibody formation *in vivo*. Thus, the *S. enteritides* is the most effective elicitor of antibody response, although both the *S. typhosa* and *E. coli* endotoxins also are effective antigens (21,22). The *S. enteritides* was better than *E. coli* which in turn was more effective than *S. typhosa* in eliciting *in vitro* lymphocyte transformation. In contrast to the above, the Piromen is a poor antibody producer *in vivo* (23). *In vitro,* it stimulated only a slight lymphocyte transformation response on one occasion. It was cytotoxic at all concentrations except the lowest. Pyrexal, which, according to Dr. S. Wolfe,†† is nonantigenic *in vivo*, did not elicit any lymphocyte transformation response, and was even more cytotoxic than the Piromen.

There are a number of possible explanations for the increased proportions of monocytes present in those cultures in which significant lymphocyte transformation also occurred. It is unlikely that this is due to mistaken identification since lymphocytes do not give rise to phagocytic mononuclear macrophages in these cultures(12,20). The increased numbers of monocytes in the cultures may somehow be related to the ability of endotoxins to increase the numbers and activity of the RES cells *in vivo*. However, there is no evidence that the endotoxin stimulated the monocytes to proliferate, because none of them labelled with H^3TdR on the fifth day in our cultures, nor have they done so in other studies of PHA stimulated WBC cultures over a period of 3 days(24). The most likely explanation is that endotoxins were toxic to granulocytes and perhaps to some small lymphocytes, and thereby allow

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monocytes to thrive and selectively survive in these cultures.

Summary. The response of peripheral WBC cultures to various endotoxins was compared to unstimulated control cultures. Three normal subjects with low antibody titers to *S. typhosa, S. enteritides* and *E. coli* were used as cell donors. These endotoxins produced lymphocyte transformation, DNA synthesis as measured by TdR-H^3 incorporation, occasional mitoses, and enhanced monocyte survival in their *in vitro* WBC cultures. In contrast, endotoxins derived from *pseudomonas* and *S. abortus equi* which are relatively nonantigenic were toxic when added to leukocyte cultures and resulted in little or no lymphocyte proliferation.

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Isogeneic Marrow Infusion Following Nitrogen Mustard in the Dog.* (30034)

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In several species it has been demonstrated that death from marrow failure following lethal irradiation can be prevented by an infusion of isogeneic marrow cells(1). Studies on the dog have shown that one billion or more nucleated autologous marrow cells following 1200 r whole-body irradiation results in sur-

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