TAMe, CTN) but is incapable of hydrolyzing sensitive bonds on larger molecules (*i.e.*, fibrinogen). Alternatively, these enzymatic activities of thrombin may be associated with different "binding sites" if not different molecules.

Summary. Preparations of alpha-2 trypsin-binding macroglobulin purified from Cohn Plasma Fraction III-O have been found to bind human and bovine thrombin. The enzyme-macroglobulin complex formed is inactive against fibrinogen but readily hydrolyzes synthetic substrates such as N-p-tosyl-L-arginine methyl ester and N-carbobenzoxy-L-tyrosine p-nitrophenyl ester. Inactivation of thrombin clotting activity by binding to the macroglobulin follows an apparent bimolecular reaction. The complex has been isolated by Sephadex G-100 gel filtration and partial success has been obtained in dissociating the esterase activity but not the clotting activity from the macroglobulin. These findings may have important implications concerning the "binding" or "active site" of the estero-proteolytic activities of thrombin as well as the role which the enzyme-macroglobulin complex may have in blood clotting.

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Endotoxin Cytotoxicity: Role of Cell-Associated Antibody.* (30801)

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The ability of bacterial endotoxins to damage cells has been demonstrated both *in vivo*(1) and *in vitro*(2,3). It has been suggested (*cf.* 3) that many of the biological effects of endotoxins, including the enhancement of host resistance to a large variety of microbial pathogens, may result from the release of intracellular materials from certain cells, such as macrophages, following their exposure to endotoxin. Among the active materials released may be oligodeoxyribonucleotides since it has been demonstrated that these oligomers, resulting from the enzymatic digestion of DNA, stimulate both antibody production, as measured by the Jerne technique(4), and phagocytosis, as measured by carbon clearance(5).

A test system(6) which utilizes monolayer cultures of peritoneal macrophages and permits a quantitation of the cytotoxicity of endotoxin *in vitro* has been developed. This test system has now been employed to de-

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termine whether the presence, on the surface of macrophages, of pre-existing antibody specific for endotoxin is required to initiate cytotoxic effects. If such antibody is required, a possible conclusion would be that the cytotoxicity of endotoxin can be a consequence of antigen-antibody reactions occurring at cell surfaces(3,7).

Two experimental approaches have been employed in studies with guinea pig macrophages. The first utilized a specific anti-γ-globulin serum to determine whether the reaction between endotoxin and a presumed cell-associated antibody against endotoxin could be blocked, thus preventing the cytotoxic effects of endotoxin. (The anti-γ-globulin employed was directed towards guinea pig γ-globulins in general rather than against anti-endotoxin specifically.) The second approach was based upon the supposition that, if pre-existing antibody to the endotoxin plays a role in cell damage, then cells from animals previously unexposed to the endotoxin employed should not be damaged by addition of this particular endotoxin *in vitro*.

Materials and methods. Male guinea pigs (Hartley strain), weighing about 350 g, were used as macrophage donors throughout. In some of the experiments, the donors were first infected intramuscularly with 1×10^3 to 1×10^5 *Brucella abortus*, SA-S, suspended in 0.2 ml of 1% (v/v) tryptose broth in saline. Endotoxins from *Escherichia coli, Serratia marcescens* and *Brucella abortus* were obtained from Difco Laboratories, Detroit, Mich. Anti-guinea pig and anti-human γ-globulin sera, prepared in rabbit or goat, were obtained from Microbiological Associates, Bethesda, Md. Small Leighton tubes (1 sq cm floor area) were obtained from Bellco Glass Co., Inc., Vineland, N. J.

A detailed description of the procedure used for estimation of cytotoxicity has been given previously(6). Peritoneal macrophages were collected, washed, placed in Leighton tubes, and exposed to endotoxin (1 to 2 γ/ml) for one hour. The monolayer which formed during this period was washed, reincubated in Scherer's maintenance solution (SMS) at 37°C for 5 to 8 hours, and then exposed to 0.5% Trypan Blue to determine the proportion of damaged cells. The total number of cells, stained plus unstained, was the same in endotoxin-treated and control cultures, thus allowing direct comparison of the percentages of Trypan Blue-positive cells. At least 3, and often 5, replicates were employed for each test condition.

TABLE I. Effect of Pretreatment of Macrophages with Goat Anti-Guinea Pig Gamma Globulin or with Goat Anti-Human Gamma Globulin Serum (5%) on Subsequent Endotoxin Cytotoxicity *in vitro*.

Pretreatment during 1st hr	% of Trypan Blue-positive cells in cultures exposed during 2nd hr to	
	SMS	Endotoxin*
SMS	8.8	57.7
Anti-g.p. γ-globulin	6.1	15.0
Anti-human γ-globulin	7.0	53.4

* *S. marcescens:* 1 μg/ml.

In experiments employing anti-γ-globulin, macrophages were exposed for one hour to the antiserum (1% to 10% in SMS), and then washed prior to the addition of endotoxin. Anti-human γ-globulin serum served as a control.

Results. As in prior studies(6), endotoxin-exposed macrophage populations showed a significantly higher percentage of Trypan Blue-positive cells than unexposed control cultures. However, when macrophages were treated with goat anti-guinea pig γ-globulin prior to their exposure to endotoxin, there was an interference with this cytotoxic effect. These results are illustrated in Table I. Data in this Table also demonstrate that pretreatment of macrophages with a control serum (goat anti-human γ-globulin) had no effect upon the subsequent cytotoxic effect of endotoxin. Similar results were obtained using rabbit anti-γ-globulin sera. It should be noted that pretreatment with either of the anti-γ-globulin sera, in the absence of subsequent exposure to endotoxin, and at the concentrations used, had no discernible effect on the cells; *i.e.*, the percentage of Trypan Blue-positive cells was the same whether or not the cells had been exposed to anti-γ-globulin sera.

In surveying endotoxins prepared from a variety of genera of Gram-negative bacteria, it was established that endotoxin from *Bru-*

TABLE II. Effect of Endotoxins from *E. coli* (O126:B6) and *B. abortus* (447722) on Macrophages from Normal and Brucella-Infected Guinea Pigs.

	Normal guinea pigs			Brucella-infected guinea pigs			
	% of Trypan Blue-positive cells; cultures exposed during 1st hr to			% of Trypan Blue-positive cells; cultures exposed during 1st hr to			
Exp No.	SMS	*E. coli* LPS*	*Br. abortus* LPS*	SMS	*E. coli* LPS*	*Br. abortus* LPS*	Agglut. titer
1	23	61	26	6	52	26	1:160
2	9	59	14	5	74	60	1:320
3	5	87	10	9	74	79	1:1280
4	5	68	4	4	61	27	1:640
5	6	35	9	10	54	76	1:5120
6	27	90	30	18	86	87	1:1280

* Concentration: 2 μg/ml SMS.

cella abortus differed from others in being non-toxic in the cell culture system employed (Table II; Normal guinea pigs). However, the same *B. abortus* endotoxin did show cytotoxic activity when macrophages were taken from brucella-infected guinea pigs (Table II). These animals had been infected 9 to 27 weeks previously and their brucella-agglutinin titers are shown in the Table. Preliminary observations indicate that the susceptibility of peritoneal macrophages *in vitro* to endotoxin from *B. abortus* requires the exposure of the macrophage donor to living *B. abortus*. Formalin-killed *B. abortus* organisms appear incapable of producing the shift from insusceptibility to susceptibility to brucella endotoxin. It should be noted that such a killed brucella vaccine is also unable to elicit lasting protection against subsequent challenge with virulent brucellae. Some efforts have been made to determine the time after exposure to living *B. abortus* when the macrophages become susceptible *in vitro* to brucella endotoxin. The time interval appears to be between 10 and 20 days.

Discussion. These data suggest that a necessary prerequisite for the cytotoxic action of endotoxin is the presence of endotoxin-specific antibody at the surface of macrophages. Inhibition of cytotoxicity by anti-γ-globulin may be interpreted as being due to the blocking of a specific cell-associated antibody which must react with endotoxin in order for cell damage to occur It is of interest to note that cell damage does not result from the globulin-anti-globulin interaction.

Whereas the results with the anti-γ-globulin cannot exclude possible direct effects of the antiserum on the tested cells, the results with *B. abortus* endotoxin lend support to the conclusion that cell-associated antibody is involved. The tests with *B. abortus* show that prior sensitization of the cell donor is a prerequisite for the cytotoxic effect of endotoxin. This conclusion is supported by the related finding that *B. abortus* endotoxin, in contrast to endotoxins from ubiquitous sources, does not produce typical toxic effects in mice(8). These data strengthen the belief that many of the biological effects of endotoxin *in vivo* are elicited as the result of damage caused by antigen-antibody reactions at the surface of certain cells. Such damages, in turn, probably result in the release of materials that have been shown to enhance both antibody production(4) and phagocytosis(5).

The interpretations advanced here do not necessarily mean that endotoxin can be cytotoxic *only* by virtue of its antigenic nature. It is quite likely that primary toxicity also exists and that cell damage can be a combination of direct toxicity and immunological events.

Summary. Cytotoxic effects of endotoxins *in vitro* seem to require the presence of endotoxin-specific antibody at the surface of susceptible cells. This conclusion is based on the finding that exposure of guinea pig macrophages to anti-guinea pig γ-globulin interferes with the cytotoxic effects of subsequent exposure to endotoxins from a wide variety of Gram-negative bacteria. The conclusion is also supported by the finding that endotoxin

from *B. abortus*, a non-ubiquitous Gram-negative organism, is not cytotoxic *in vitro* unless macrophage donors have first been sensitized by infection with live *B. abortus*.

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Prevention by Methionine Feeding of Atherosclerosis and Thrombosis in Hyperlipemic Rats.* (30802)

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It has been observed in species such as rat (1,2), fowl(3,4) and man(5,6) that the serum cholesterol level can be markedly decreased by increasing the dietary level of protein. As a result, it was possible to suppress atherosclerotic lesions in chicken by feeding high protein diets(3) and it may be that the intake of animal protein also accounts for unexplained variances in the death rate of man from atherosclerosis(7). This effect of proteins could be ascribed to their content in sulfur amino acids, since methionine has been shown to exert hypocholesterolemic effects in the rat(1,2) and the monkey(9), and even to inhibit atherosclerosis in the latter(9).

A recent study from this laboratory(10) reported that in hooded rats fed certain hyperlipemic diets a marked reduction in the cholesterolemia, the degree of atherosclerosis and the incidence of disseminated thrombosis could be obtained by increasing the dietary level of casein. The purpose of the present experiments was to investigate whether this preventive effect of casein could be attributed to its methionine content. The effect of this amino acid was compared with that of others and also of choline, which is known to be as effective as methionine in preventing hepatic lipidosis and necrosis(11). Although no definite relationship appears to have been established between fatty infiltration of the liver and atherosclerosis, rats, like other species, exhibit marked hepatic fatty changes when fed high-fat diets to produce early lesions of atherosclerosis.

Materials and methods. A total of 132 male hooded rats (Quebec Breeding Farm, St. Eustache, Qué.) with an initial body weight of 130-140 g were utilized for this study. At the beginning of the experiment each group comprised 12 rats, except Group 1, which contained 24 rats. When the first experiment had been terminated, Groups 1, 2 and 6 were repeated under the same conditions with 12 rats per group and the results listed in the Table constitute the data obtained from both sets of observations. For various reasons, several animals were eliminated in the course of these experiments and the number of animals actually utilized in each group is listed in the Table.

The rats were housed 6 per cage and given, *ad libitum*, tap water alone or, from time to time when deemed necessary, tap water to which was added 187.5 mg per 1,000 ml of oxytetracycline to prevent pulmonary infections.

The basic diet given as such only to Group

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