ABSENCE OF A DEMONSTRABLE EFFECT FOR ENDOTOXIN ON THE LYSOSOMAL ENZYME ACTIVITY OF RECRUITED MACROPHAGES IN A RAT MODEL OF LIVER INJURY

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INTRODUCTION

Endotoxin has been implicated in the pathogenesis of a number of acute and chronic liver diseases(3,11,14). In a C.parvuminduced animal model of liver injury(8) it has been proposed that recruited and activated tissue macrophages are sensitive in vivo to intravenous administration of endotoxin; they have postulated that the activated macrophages release factors toxic for hepatic parenchymal cells following exposure to endotoxin. It has been shown in other experimental situations that bacterial endotoxin can stimulate a variety of macrophage secretions (1). Chronic inflammation may be promoted by lysosomal enzyme release(6), and we have therefore measured a single lysosomal enzyme (NAG) as a marker of this activity. In other experimental situations, activated macrophages have

been shown to be sensitive to low concentrations of endotoxin (17). In this study, activated and recruited liver macrophages have been isolated from a C.parvum-induced animal model of liver injury, and their endotoxin does response(concentration range 5 ng.-50 µg./ml.)measured with respect to their production of a lysosomal enzyme. The aim of this study was to establish whether promotion of hepatic damage through endotoxin administration might occur through enhanced macrophage release of lysosomal enzymes.

METHODS

Animals. Adult female Wistar rats weighing 250-300 gms. were used in all studies. A model of liver injury was established by injecting these rats with killed corynebacterium parvum (Wellcome). One ml. of a 7mg/ml. suspension was given via the tail vein six days before sacrifice. In addition to the cell isolation procedures described below some hepatic specimens were kept and processed for histology.

Cell isolation procedure.Liver macrophages were isolated using a method described in detail elsewhere[5,7]with minor modifications as indicated below. The rats were anaesthetised with ether and, following a midline abdominal incision, the portal vein cannulated. The liver was perfused in situ for two minutes with 0.2% Pronase AS(Protease V Sigma) in Hank's balanced salt solution (HBSS) containing 100iu/ml. Benzyl penicillin, 100 µg/ml.Streptomycin and 250µg/ml. Amphotericin B. The liver was 'then removed, weighed and finely minced. The fragments were digested for one hour with constant stirring in 90mls

of 0.2% pronase solution maintained at a pH of 7.35 $\pm$ 0.
05 and a temperature of 37°C. The pH was adjusted as ne-
cessary with the addition of 0.5 N sodium hydroxide. At
15 and 40 minutes after the start of the incubation.0.5
mg. DNA ase (Type I, Sigma) in 1ml. HBSS was added to
aid digestion of -cllular debris. This has been shown to
improve subsequent cell resuspension and yield(7).Steril-
ised glassware was used throughout.

Following incubation the suspension was allowed to
stand for two minutes to allow large debris to sediment;
the resulting suspension was washed three times in HBSS.
This was then layered on Ficoll-Hypaque(Pharmacia)and sp-
un for 15 minutes at 600g. The interface layer of cells
was harvested and washed once in HBSS. The total number
of cells was then counted, their viability determined by
Trypan blue exclusion and a cytocentrifuge preparation
was stained for esterase. The macrophage population from
this non-parenchymal cell preparation was purified by ad-
herence. 6 x 10^6 non-parenchymal cells in 2mls. of RPMI
1640 (Gibco)containing antibiotics and 0.05% Lactalbumin
Hydrolysate (Sigma)were added to 35 x 10mm. multiwell tis-
sue culture plates(Linbro).Up to twenty-four separate wel-
ls were established for each separation. The cells were
incubated overnight at 37°C in a fully humidified incuba-
tor at 5% CO_2. The non-adherent cells were then removed
by gentle washing with HBSS and the purity of the adhere-
nt population assessed using latex particle phagocytosis.
Phagocytosis. Sterile glass cover slips were placed in
two wells of the multi-well plates before addition of
the non-parenchymal cell suspension. The macrophages were
allowed to adhere overnight to the cover slips;non adher-

ent cells were then removed by gentle washing and 0.81µ.
latex particles (Difco)added to the adherent cells in cu-
lture medium at an approximate particle to cell density
of 50:1. These were incubated for a further hour,the co-
ver slip removed, mounted and sealed and the cells obser-
ved using phase contrast microscopy(x80). Cells contain-
ing more than three latex particles were scored as positive.

Adherence.After overnight culture the supernatant from
one well was gently agitated to suspend all non adherent
cells and a cell count performed on an aliquot taken fr-
om the supernatant. The supernatant was then discarded
and to the adherent cells remaining in the well after one
wash with HBSS was added 1ml. of 0.7% lignocaine in RPMI.
After incubation at 4°C. for 15 minutes, the well was gen-
tly scraped with a rubber policeman and a cell count per-
formed on the resulting cell suspension. Adherence was
expressed as the percentage of adherent cells retrieved
compared to the total number of cells retrieved after
overnight culture.

Esterase staining.Cytocentrifuge preparations of non-par-
enchymal cells were stained for non-specific esterase,
using the method as described by Horwitz et al.(12).

Culture and stimulation of macrophages. Following the
purification of the adherent population of cells from
the normal rats, the media was changed and culture con-
tinued for a further 24 hours. The cells isolated from
the experimental model were exposed to endotoxin for two
hours before changing media and subsequent 24-hour cul-
ture. Lipopolysaccharide(Salmonella typhosa 0901, Difco)
was added in suspension to duplicate wells at appropria-
te concentration, so that the cells were exposed to the

following concentrations of endotoxin: 0,5 ng., 50 ng., 500 ng., 5 µg. and 50 µg./ml.

At the end of the culture period, supernatants were collected from each well. The adherent cells were removed from the plastic surface of each well with a rubber policeman' into sterile, cold, distilled water and the cell suspension was rapidly frozen and thawed six times. The supernatants and lysates were stored at -20°C. for enzyme assay. Assays were completed within two weeks of this collection. All experiments were performed in duplicate. The total protein concentration of the cell lysates was estimated by the method of Lowry et al.(13).

N-acetyl-B-glucosaminidase assay (NAG). A sensitive assay for NAG activity in the cell lysate and supernatant was developed from the spectrofluorometric method of Beutler et al.(2). The incubation mixtures contained 0.5 ml. of a 3 mmol./litre solution of 4-methyl-umbelliferyl-D-glucopyranoside (Koch-Light Lab.) in 0.05 mol./litre citrate buffer(pH 4.5). 100 µl. of either cell lysate or supernatants were added to the incubation mixtures and the 37°C. incubation terminated at the end of one hour by the addition of 2.5 ml. glycine buffer (pH 10.5). All sample assays were performed in duplicate. A standard curve was constructed using known concentrations of 4-methyl-umbelliferone(Koch-Ligh Lab.). Interassay variation was less than 10%.

Statistics. Significant differences were established using a test with significance concluded at a probability of less than 5%.

RESULTS

Animal model. The histology of the rat livers six days

following an intravenous injection of Corynebacterium parvum is illustrated in Figure 1. A heavy mononuclear cell infiltrate is present in the portal tract,epithelioid cell granulomas are distributed throughout the lobule and there is an increase in cells lining the liver sinusoids. A minor degree of hepatocyte damage has occurred and we have shown that this damage is greatly enhanced by subsequent intravenous administration of endotoxin. Our findings are similar to those described by Ferluga and Allison(8).

Cell isolation. There was a fourfold increase in non-parenchymal cell yield from the C.parvum-injected rats(Table 1), and this is reflected in the histological changes. The results for the non-parenchymal cell suspension of normal rats are in close agreement with those of other workers(7). Viability was 89 $\pm$ 2% for the cells isolated from normal rats, 93 $\pm$ 2% for cells from C.parvum-injected rats; esterase positivity (macrophages and endothelial cells)was similar for the two groups of cells. The number of liver macrophages obtained was derived from the proportion of adherent cells that were actively phagocytic after overnight culture(see Table 1).The yield of macrophages established in culture per gm. liver weight was 3.1 $\pm$ 0.2 x 10^6 for normal rats and 11.9 $\pm$ 0.9 x 10^6 for C.parvum- injected rats. The proportion of lymphocytes in the non-parenchymal cell suspension was greater from the C.parvum-injected rats(24%)compared to the normal rats(4%). The number of endothelial cells isolated from the experimental model is also increased. Thus, the proportion of macrophages isolated from both groups did not differ, but the absolute numbers from C.

parvum livers were increased.

Enzyme production, N-acetyl-B-glucosaminidase(NAG) and endotoxin dose response of recruited cell. NAG cell lysate and supernatant activity was significantly higher(p$<$ 0.02: see Figure 1) in the cells derived from the C.parvum-injected rats as compared to normal (normal cells; supernatant activity 0.09 $\pm$ 0.02 nmol. product/μg. cell protein, cell lysate 0.15 $\pm$ 0.04 nmol. product/μg. cell protein; C.parvum-recruited cells supernatant activity 0.19 $\pm$ 0.03 nmol. product/μg. cell protein cell lysate 0.41 $\pm$ 0.07 product/μg. cell protein, mean $\pm$ sem). On exposure of the cells derived from the C.parvum-injected rats (n = 6) to a wide range of endotoxin concentrations, no alteration in NAG supernatant or cell lysate activity was observed (Table 2).

DISCUSSION

It has been proposed that lysosomal enzymes released by recruited and activated tissue macrophages may lead to tissue injury (6).A variety of immunological stimulants, including immune complexes, lymphokines, complement components, zymosan and bacterial products, are able to induce release of these hydrolytic enzymes from cells of the macrophage-monocyte system(1,4,15,16). In this study we have observed a significantly increased NAG activity in both supernatants and cell lysates of cells recovered from the C.parvum-injected animals. These changes probably reflect activation of these cells in vivo. Whether the increased NAG levels to which the hepatocytes are exposed result in cellular damage remains to be determined. The potential exposure to NAG of the hepatocytes in the expe-

rimental model is increased by a factor of twelve,since
not only is the specific NAG activity increased but also
the absolute macrophage numbers. The role of endotoxin
in enhancing macrophage activity has been studied in a
variety of situations. Peripheral monocytes from subjec-
ts with rheumatoid arthritis, inflammatory bowel and ch-
ronic liver disease have been shown to produce more lyso-
somal enzymes when cultured in vitro following endotoxin
stimulation, as compared to normal peripheral monocytes
(9). Furthermore, it has been suggested that recruited
and activated liver macrophages, isolated from Corynebac-
terium parvum-primed mice, are more sensitive than macro-
phages from non-primed animals in response to circulating
endotoxin, and that subsequent release of toxic products
leads to liver injury (8). The cytotoxicity of activated
macrophages has been shown to be enhanced by very low co-
ncentrations of endotoxin (0.5-lng./ml.), whereas the cy-
totoxicity of non-activated macrophages remains uninflue-
nced by amounts of up to 10 µg.ml. of endotoxin(17). We
have shown that the cell lysates of liver macrophages is-
olated from a C.parvum rat model of liver injury have si-
gnificantly increased lysosomal enzyme activity after 24
hours in in vitro culture when compared to normal resid-
ent liver macrophages. Endotoxin concentrations in a ran-
ge from 5 ng. to 50 µg./ml. produced no significant incr-
ease in either cell lysate or supernatant NAG activity
of recruited and activated liver macrophages isolated fr-
om the experimental model. Significant differences were
not observed when compared to the activities of cells un-
exposed to endotoxin. We interpret these results as show-
ing that the recruited and activated macrophages in this

model of liver injury are already maximally stimulated in vivo. Subsequent in vitro exposure to endotoxin in varying concentrations has not resulted in further increase in NAG activity. There was no apparent sensitivity to low concentrations of endotoxin which might be circulating undetectable by the limulus assay. Although endotoxin has been implicated in the pathogenesis of certain liver disease,we have not been able to demonstrate in this model that mediation through an increase in lysosomal enzyme release by activated macrophages occurs.However, it is possible that the release of other macrophage secretory products is stimulated further in the presence of endotoxin (10).
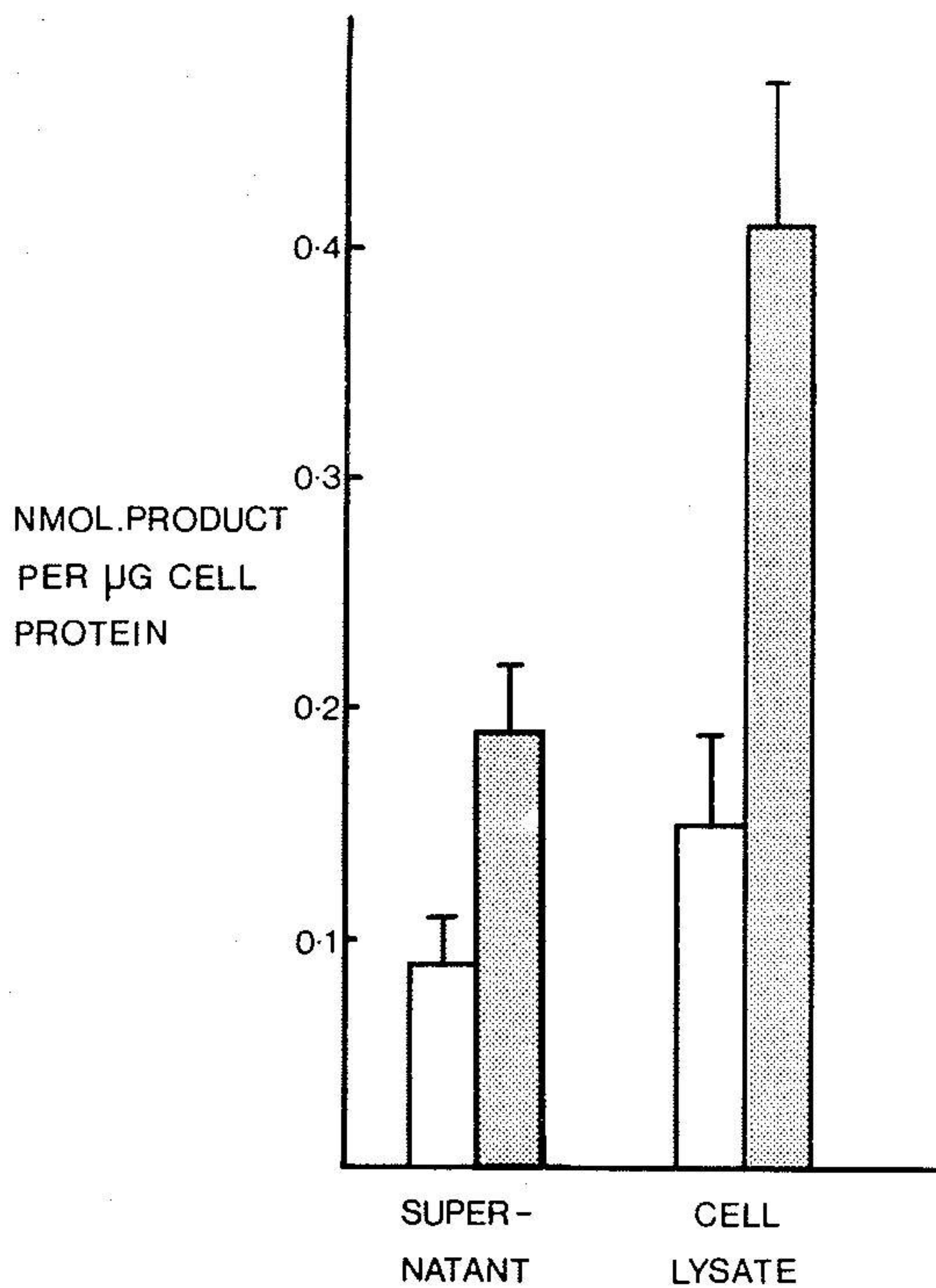
Figure 1.

N-acetyl-B-glucosaminidase activity in cell lysates and
supernatants of both cells from normal rats (n = 10; un-
hatched) and cells isolated from C.parvum-injected rats
(n = 14, hatched)

TABLE 1.

Cell Isolate Results

	Normal rats n = 10	C.parvum injected rats n = 14	Significance (independent t=test)
Cell yield/gm. digested liver wt. x 10^6.	10.9±0.8	41.6±6.9	p < .001
Viability.(%)	89±2	93±2	n.s.
Esterase[1] staining.	71±4	70±3	n.s.
Adherence after overnight culture.	32±8	34±6	n.s.
Phagocytic index[2] of adherent cell population.	89±3	84±4	n.s.
Liver macrophage numbers yield/gm.[3] digested liver wt.x 10^6.	3.1±0.2	11.9±0.9	p < .001

All results expressed as mean + s.e.m.

1- Figures represent percentage positive(macrophages plus endothelial cells).

2- Represents the percentage of cells actively phagocytic at time of assessment.

3- Derived from adherence and phagocytosis figures.

Table 2

N-acetyl-B-glucosaminidase activity of cell lysates and
supernatants of recruited liver macrophages after 24 ho-
urs in in vitro culture following exposure to varying co-
ncentrations of endotoxins(mean $\pm$ s.e.m.)n=6; macrophages
from separate animals were established in 12 different
wells and exposed to the different endotoxin concentrati-
on in duplicate.

Endotoxin concentration	Cellular activity nmol.activity/µg.cell protein	Supernatant activity nmol.activity/ µg.cell protein
Nil	0.46 $\pm$ 1.16	0.16 $\pm$ 0.0w
5 ng.	0.42 $\pm$ 0.05	0.14 $\pm$ 0.03
50 ng.	0.43 $\pm$ 0.09	0.11 $\pm$ 0.03
500 ng.	0.35 $\pm$ 0.04	0.10 $\pm$ 0.02
5 µg.	0.47 $\pm$ 0.15	0.12 $\pm$ 0.05
50 µg.	0.38 $\pm$ 0.14	0.10 $\pm$ 0.04

SUMMARY

The endotoxin dose response of recruited and activated
tissue macrophages isolated from a rat model of liver
injury has been measured after 24 hours in in vitro cul-
ture, with respect to their production of a lysosomal en-
zyme, N-acetyl-glucosaminidase (NAG). There were no sig-
nificant differences in the NAG activity in cell lysates
or supernatants following a two-hour exposure of the ma-
crophages to a range of endotoxin concentrations (50 µg.

5 ng./ml.); nor were any significant differences observed when compared to NAG activity of cells recruited but not exposed to endotoxin. The level of NAG activity in the supernatants and cell lysates of these recruited cells was significantly higher (p $<$ 0.02) than the levels measured in resident macrophages (Kupffer cells) isolated from normal rat livers.

We conclude that with respect to lysosomal enzyme release these cells have already been maximally activated in vivo and that further hepatic damage induced by endotoxin administration occurs through mechanisms other than increased release of lysosomal enzymes.

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