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LYMPHOCYTE-SALMONELLA INTERACTION: ENERGY DEPENDENCE AND THIOL GROUP INVOLVEMENT

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SUMMARY : In order to gain better insight into lymphocyte-Salmonella interaction investigation has been carried out on energy-dependence and involvement of thiol groups in this process by using a modified rosette test. Binding frequency, number of bound bacteria/number of binding lymphocytes and the number of bacteria-binding sites/lymphocyte were found to be enhanced by externally added ATP and decreased by both uncouplers and electron transfer chain inhibitors. Treatment of either bacteria or lymphocytes with thiol reagents, such as mersalyl or N-ethyl-maleimide, prevents lymphocyte-Salmonella adherence, thus showing the presence of thiol groups involved in the binding mechanism in both bacteria and cells. Consistently, as a result of mersalyl inhibition, a decrease in the number of bacteria-binding sites/lymphocyte was also found.

Modification of the bacterial rosette test used so far to show spontaneous bacteria-lymphocyte binding (1-3) has allowed quantitative investigation of lymphocyte-Salmonella interaction. The frequency of binding lymphocytes was found to depend on both incubation time and Salmonella/lymphocyte ratio, thus favouring the potential ability of lymphocytes to modulate the immune response during the course of bacterial infections (4). Moreover, the occurrence of saturation kinetics confirmed the existence of receptor sites on the cell surface able to bind bacteria (1,4). Nevertheless, since the mechanism by which lymphocytes can bind bacteria still remains rather obscure, this paper investigates two aspects of bacteria-lymphocyte binding: energy dependence and involvement of thiol groups in the process.

Abbreviations: CCCP = Carbonyl Cyanide m-Chlorophenyl-hydrazone; HBSS = Hank's Salt Balanced Solution; NEM = N-ethyl-maleimide; PBL = Perypheral Blood Lymphocytes; PBS = Phosphate Buffered Saline solution.

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MATERIALS and METHODS

Rotenone, antimycin A, sodium azide, oligomycin, CCCP, mersalyl, NEM and cysteine were from SIGMA.

Bacterial strain. *Salmonella* minnesota Rb was grown according to (1).

PBL isolation. This was achieved as previously described (5-6).

Assay of Salmonella binding to PBL. In a typical experiment PBL ($5x10^5$ cells) were incubated in Eppendorf cups in medium standard (PBS), consisting of 0.15 M NaCl, 0.02 M phosphate buffer pH 7.20 at 20° C. Aliquots of *Salmonella* were then added in the absence or presence of different compounds. The final volume was usually kept equal to 0.5 ml. Cell viability was assessed by Trypan blue stain technique. The lymphocyte-bacteria reaction was stopped at different times by rapidly centrifuging the samples in a Haeraeus Christ microcentrifuge equipped with an Eppendorf rotor (10000 rpm for 30 sec). The supernatant was immediately discarded and the pellet resuspended in 0.2 ml of HBSS and examined by phase-contrast microscopy.

The binding parameters were measured as previously shown (4): in short, only *Salmonella* attached on the round surface of each lymphocyte were considered to describe quantitatively bacteria-cell interaction.

The parameters used in this paper are defined as follows:

BL (Binding Lymphocytes) = bacteria binding lymphocytes/counted lymphocytes x 100.

BB (Bound Bacteria) = number of total bound bacteria/number of binding lymphocytes.

It should be noted that according to its definition minimum BB will be 1; thus, in the case of BB stimulation or inhibition, to illustrate better experimental findings, BB-1 Control% will be also reported.

FB (free bacteria) = difference between the number of added bacteria/binding lymphocyte and the number of bound bacteria/binding lymphocyte.

BBS (Bacteria Binding Sites) = number of bacteria-binding sites/lymphocyte.

In different experiments significant changes in binding parameters were found, likely due to lymphocytes obtained from different healthy donors.

RESULTS

Energy dependence of lymphocyte-Salmonella binding. The effect of ATP (used as an energy source) on BL and BB in the absence and presence of oligomycin, an inhibitor of mitochondrial ATPase, is shown in Table 1 (Exp. I). Externally added ATP (2 mM) increases both binding frequency (BL) and the capacity of each binding lymphocyte for *Salmonella* (expressed by BB-1) (63% and 40%, respectively). Such an increase is prevented by oligomycin, which *per se* slightly reduces BL, but significantly inhibits BB. This result favours energy dependence of lymphocyte-*Salmonella* binding.

In order to substantiate this conclusion further, the effect of the uncoupler CCCP as well as of the electron transfer chain inhibitors on both BL and BB was also investigated (Table 1, Exp. II). In this case the tested compounds were allowed to incubate with lymphocytes for 30 min before the addition of bacteria. CCCP (10 μM), rotenone (10 μg), antimycin (10 μg) and sodium azide (10 mM) were found to inhibit strongly or completely both BL and BB, thus clearly showing that lymphocyte-*Salmonella* binding is an

TABLE 1

EFFECT OF ATP, OLIGOMYCIN AND ELECTRON TRANSFER CHAIN INHIBITORS ON LYMPHOCYTE-SALMONELLA INTERACTION

Test substance and quantity	BL	Control %	BB	BB-1 Control %
Exp. I				
None	35	100	2.0	100
ATP (2 mM)	57	163	2.4	140
Oligomycin (2 µg)	32	91	1.7	70
Oligomycin + ATP	30	86	1.8	80
Exp. II				
None	30	100	1.9	100
CCCP (10 µM)	7	23	1.4	37
Rotenone (10 µg)	5	17	1.4	37
Antimycin (10 µg)	8	27	1.0	0
Sodium azide (10 mM)	3	10	1.0	0

Lymphocytes ($5x10^5$ cells) were incubated in 0.25 ml of PBS for 2 min (Exp. I) or 30 min (Exp. II) in the absence or presence of the indicated compounds. Salmonella ($8x10^6$ cells) were added and the binding reaction stopped after further 30 sec. Both BL and BB were determined as described in the Methods. In Exp. I, line 4, ATP was added 2 min after oligomycin and 2 min later Salmonella were added.

energy-dependent process. Control was made that the used compounds had no effect on cell viability by using Trypan blue stain technique. It should be noted that in this experiment use was made of inhibitor concentrations much higher than those able to inhibit energy metabolism of both isolated rat liver and lymphocyte mitochondria (7). However, in other experiments (not shown here) lower inhibitor concentrations were used (about ten times less than those in Table 1) with preincubation time ranging from 2 and 60 minutes. While BL inhibition increased progressively with an increase in incubation time (for instance when rotenone was used, 33% and 67% inhibition was found after 2 or 60 min incubation respectively), on the contrary BB-1 inhibition (50% for rotenone) did not change significantly with time.

The number of BBS in the absence or presence of ATP, CCCP and sodium azide was determined by means of the Scatchard plot, in which the ratio of BB/FB was plotted v.s. BB (Fig. 1). In all cases straight lines were obtained according to linear regression analysis of the experimental data. The number of BBS given by the intercept on the horizontal axis was found to increase

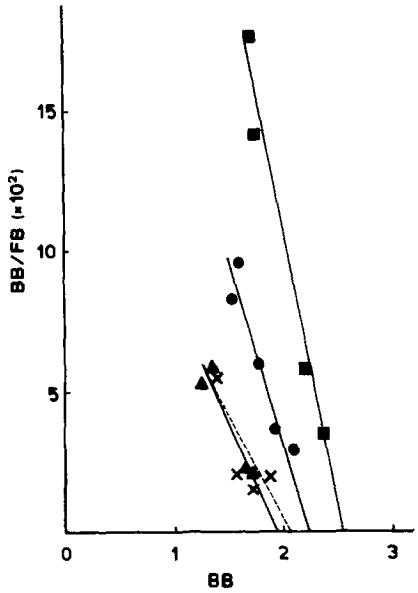

Fig.1 The effect of ATP, CCCP and sodium azide on lymphocyte bacteria-binding sites measured by means of the Scatchard plot. PBL (5 x 10^5 cells) were incubated for 2 min in the absence (●) or presence of ATP (2 mM) (■), CCCP (10 μM) (▲), and sodium azide (10 mM) (✕). Different aliquots of Salmonella were then added to obtain Salmonella/lymphocyte ratios ranging between 4 and 20. The binding parameters were measured as described in the Methods and plotted according to (4). Linear regression analysis of the experimental results gave the reported straight lines whose intercepts at the abscissa axis represent the BBS values. Correlation coefficient values were found to be 0.93 (●), 0.96 (■), 0.86 (▲) and 0.74 (✕), respectively.

owing to ATP (2 mM) addition (from 2.25 to 2.55), but decrease to 1.95 and 2.05 in the presence of CCCP (10 μM) and sodium azide (10 mM) respectively.

The effect of thiol reagents on lymphocyte-Salmonella interaction.

Experiments on the effect of thiol reagents on lymphocyte-Salmonella binding were carried out using two different compounds: mersalyl which reacts with thiols by virtue of the presence of a mercury atom and NEM, which contains a susceptible carbon-carbon double bond. Both reagents have been extensively investigated with respect to inhibition of processes such as metabolite and protein transport in mitochondria (8,9).

Both BB and BL decreased when lymphocytes were incubated with Salmonella in the presence of the thiol reagents, thus showing the involvement of certain thiol groups in the process.

The dependence of BL inhibition on increased thiol reagent concentrations was also investigated (not shown here). For 2 min incubation of lymphocytes

with either mersalyl or NEM, maximum inhibition was obtained by using 1 mM concentration of both mersalyl (50 % inhibition) and NEM (70 % inhibition). However, by increasing preincubation time of lymphocytes with the thiol reagents progressive inhibition occurred (60% and 90% for mersalyl and NEM, respectively for 10 min incubation).

In order to gain futher insight into the mechanism by which thiol reagents can influence lymphocyte-*Salmonella* interaction, experiments were carried out in which either lymphocytes or *Salmonella* (Table 2) were separately preincubated with the thiol reagents prior to starting reactions. Both mersalyl and NEM-treated lymphocytes (Exp. I) displayed reduced ability to bind *Salmonella*, as shown by the decrease of both BL and BB. In the same experiment, both prevention and reversal of the thiol reagent inhibition by cysteine was also tested. 10 mM cysteine (which *per se* gives 13% BL

TABLE 2

MEASUREMENTS OF BL AND BB FOLLOWING PRETREATMENT OF EITHER LYMPHOCYTES OR SALMONELLA WITH THIOL REAGENTS

Preincubation with	Further addition of	Exp. I Control %		Exp. II Control %	
		BL	BB-1	BL	BB-1
None	None	100	100	100	100
Mersalyl	None	75	50	72	45
NEM	None	60	25	53	36
None	Cysteine	87	100	95	104
Mersalyl	Cysteine	85	96	99	91
NEM	Cysteine	68	43	65	45
Cysteine	None	87	100	98	100
NEM + Cysteine	None	85	90	95	104
Mersaly + Cysteine	None	90	98	96	98

Either lymphocytes (5 x 10^5 cells) (Exp. I) or Salmonella (2.4 x 10^8 cells) (Exp. II) were preincubated for 2 min in PBS in the absence of any compounds or presence of the indicated compounds at the following conconcentration: mersalyl 1 mM, NEM 1mM, cysteine 10mM. Additions of either PBS or cysteine (10mM) were then made. After further 2 min the exceeding reagents were removed by centrifugation and careful washing and the pellets resuspended in PBS (0.5 ml, Exp. I, 0.6 ml, Exp. II); BL and BB were determined as described in the Methods, by using either pretreated lymphocytes and Salmonella (Exp. I) or lymphocytes and pretreated Salmonella (Exp. II). In both cases 5x10^5 cells and 8x10^6 bacteria were used.
BL and BB in the absence of any compounds were 36 and 2.2 (Exp. I) and 32 and 2.0 (Exp. II) respectively.

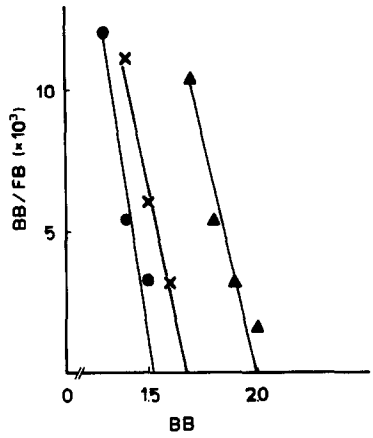

Fig.2 The effect of mersalyl on lymphocyte bacteria-binding sites measured by means of the Scatchard plot.
PBL (5 x 10^5 cells) were incubated for 1 min in the absence (▲) or presence of either 0.5 mM (✕) or 1 mM (●) mersalyl and the Scatchard plot obtained as described in Fig. 1.
Correlation coefficient values were found to be 0.94 (▲), 0.99 (✕) and 0.95 (●), respectively.

inhibition, but no BB inhibition) largely reversed mersalyl inhibition, while NEM inhibition reversal was less effective. Noteworthy prevention of inhibition was found in both cases.

When either mersalyl or NEM-treated Salmonella were used (Exp. II), both BB and BL were found to decrease. Both mersalyl and NEM inhibition were (largely or partially respectively) reversed by cysteine (10 mM), which per se had no significant effect on the control. Addition of cysteine together with the thiol reagents completely prevented BL and BB inhibition.

The effect of externally added mersalyl on the number of bacteria-binding sites/binding lymphocyte, was investigated in an experiment carried out at equilibrium by means of the Scatchard plot (Fig. 2B). As a result of mersalyl inhibition the number of binding sites (2 in the control) was decreased to 1.7 and 1.5 when 0.5 or 1 mM mersalyl respectively was used.

DISCUSSION

This paper deals with two features of lymphocyte-Salmonella binding: energy dependence and thiol group involvement. In fact, the quantitative analysis recently developed to study this process (4) allows for a detailed investigation of these features so far completely unknown.

The criteria commonly used to show the energy dependence of a biological process, namely sensitivity to compounds able to either enhance or prevent energy production (see 10-12), suggest that energy is required to induce lymphocyte-bacteria interaction. It should be noted that possible cell energization was proposed to be due to laser irradiation of lymphocytes, resulting in an increase in the binding capability (4). The energy supply appears to increase the frequency of binding cells, probably by recruiting certain lymphocyte populations otherwise ineffective in binding. Moreover, the increase in both BB and BBS suggests that energy can enhance cell capacity for bacteria through induction of new receptor sites. Consistently, the blocking of the electron transfer chain by rotenone, antimycin and sodium azide, which prevent most of cell energy generation, decreases the lymphocyte-Salmonella interaction.

In view of the absence of metabolism in the killed Salmonella used in this study, lymphocyte mitochondria appear to be the energy producing organelles. At present it is difficult to determine the nature of the physiological energy source used in the bacterial binding process (electrochemical proton gradient or ATP); therefore, the oligomycin prevention of ATP stimulation of both BL and BB consistent with a mitochondrial ATPase-linked reaction, requires further investigation. Although experimental data shown in this paper are in apparent contrast with those reported by Räsänen et al. (3) who suggested that oxidative phophorylation is not involved in the binding of lymphocytes with certain bacteria, the use of a bacteria/lymphocyte ratio of 200 in the rosette test might have prevented careful quantitative investigation.

Lymphocytes and Salmonella both appear to contain thiol groups which play an important role in the bacterial adherence to PBL and can react with mersalyl or NEM. Since mersalyl reduces the frequency of binding as well as the number of bacteria-binding sites, a possible interaction of mersalyl with thiol groups present in a proteinaceous receptor area of lymphocytes may be proposed. It should be noted that, unlike NEM, mersalyl is unable to enter mitochondria (15). Thus, assuming that this compound cannot penetrate

lymphocyte membranes either, the receptor should be located on the outer surface of the cell membrane.

As far cysteine reversal of inhibition is concerned, it is well known that NEM forms a stable covalent bond with thiols and that, unlike mersalyl, its action cannot be reversed by added thiols (see 14). Thus the partial reversal of inhibition reported in Table 2 is likely to be caused by an unknown effect of cysteine which seems to modify certain lymphocyte and Salmonella characteristics, without significantly affecting the binding properties.

As far as Salmonella is concerned, previous studies on the relationship of different Salmonella strains with lymphocytes suggested that binding is due to the interaction of bacterial lipopolysaccharide and cellular proteinaceous receptors, as evidenced by the binding inhibition observed after trypsin treatment of lymphocytes (1). However, indication is given in this paper of the existence of protein/s of the gram-negative cell wall, e.g. lipoproteins (15) which could also participate in binding.

It should be noted that, in view of the previously shown dependence of both BL and BB on the Salmonella/lymphocyte ratio (4), the reduced ability of bacteria to bind cells might also account for the decrease of these parameters.

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