NON SPECIFIC EFFECTOR-INDUCED ENZYME MODULATION IN ISOLATED PLASMA MEMBRANES

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1. Introduction

The mechanism of adjuvant activity is extremely complex, involving the antigen itself and the host [1]. It has been shown that different adjuvants may act on macrophages, B and T cells [2] and it has been observed that adjuvants can induce preferably IgM or IgG antibody stimulation depending upon the adjuvant used [3,4]. However it is difficult to understand why some adjuvants can act preferentially on one of these cells and at what level of the cell this activity is expressed. Since Concanavalin A (Con A) is known to stimulate lymphocytes [5,6] and peptidoglycans to stimulate also the immune response [7,8] we designed an in vitro system to test both adjuvants.

Since lectins, which are known to bind glycoproteins of the cell membrane surface, have in some instances adjuvants activity [6,9,10] it can been hypothetized that adjuvants could play a role at the plasma membrane level. This hypothesis is substantiated by the fact that a glycopeptide from *M. tuberculosis* which has adjuvant activity binds IgG_2 and cellular surfaces [11] and induce preferentially an IgG_2 antibody response [12]. It is also known that mitogens induce enhancement of membrane functions such as fluxes of amino-acids [13], K^+ [14,15], Ca^{++} [16] and *B. pertussis* or poly A–U can inhibit the lymphocyte response to cyclic AMP, isoproterenol or prostaglandin E_1 [17,18]. Thus one of the main problem of adjuvant activity would be to understand how adjuvants can activate the cell membrane. In order to answer this question, we should work with a pure cell population, which is not possible with the immune system, and we should avoid secondary interactions between the plasma membrane and the cytoplasm. Thus we should purify the cell plasma membrane and in a preliminary report we have shown that using a Con A-bovine serum albumine copolymer it is possible to separate inside-out (I.O.) and right side-out (R.S.O.) membrane vesicles [19]. This study was undertaken to examine the effect of two different adjuvants on the cell plasma membrane enzymatic activities: we expected to observe the stimulation of enzymes in a certain range of doses.

2. Materials and methods

2.1. *Cells*

MF_2s cells derived from the murine $MOPC_{173}$ plasmocytoma [20]. They were adapted to grow as ascitis in Balb/c mice. The preparation of the cells has been described [19].

2.2. *Plasma membranes purification*

The technique has been described elsewhere [21].

2.3. *Isolation of I.O. and R.S.O. plasma membrane vesicles*

The technique has been already described [19].

2.4. *Determination of enzymatic activities*

Enzymatic assays were performed with 66 μg of protein per ml of incubation medium. 5′-nucleotidase (EC 3.1.3.5) activity was measured by the method of Emmelot and Bos [22]. ($Na^+ + K^+$) stimulated Mg^{++}-ATPase (EC 3.6.1.3) was measured by a slight modification of the Bakkeren and Bonting method [23]. Protein concentration was estimated [24] and phosphorus measured by a modification of the Marsh technique [25].

2.5. *Sialic acid determination*

The total amount of sialic acids was estimated by the Warren technique [26]. Specific measurements of the exposed sialic acid were done by adding 50 μg of the *Cl. perfringens* neuraminidase to 1 mg of membrane protein (EC 3.2.1.18 Sigma).

2.6. *Adjuvants*

Con A from Calbiochem was used. A peptidoglycan extracted by acetylation of delipidated cells from *M. tuberculosis*, var. *hominis*, strain H_{37} Ra was finally purified by two successive filtrations on Biogel P 10 [7,27]. It has a mol. wt. around 3500 and its composition is as following (molar ratios between parenthesis): Ala (3); Glu (2); DAP (2); GlcN (2); Mur (2); this substance yet contained 20% sugars (Ara, Man, Gal).

2.7. *Labelled Con A*

[^{3}H]acetyl Con A (specific activity 4600 cpm/μg of protein) was a gift from Dr M. Monsigny.

2.8. *Labelling vesicles*

40 μg of proteins were taken for either I.O. or R.S.O. vesicles and were incubated in a buffer containing from 0.05 to 0.8 μg of labelled Con A and incubated at 37°C for 30 min. The incubated membranes were then put on a 0.22 μm Millipore filter and extensively washed. After drying, the filters were put in vials and counted in an Intertechnique scintillation counter.

3. Results

Right side-out vesicles were characterized by three criteria (table 1). The total amount of sialic acids estimated by acid hydrolysis was found to be 120 nmol per mg of protein in R.S.O. vesicles and 125 nmol per mg of protein in I.O. vesicles. Release by neuraminidase was twice higher in R.S.O. vesicles than in I.O. vesicles i.e. 110–120 nmol/mg of protein and 50–60 nmol/mg of protein respectively. Thus we can assume that the relative large amount of free sialic acids found in I.O. could be due to a partial permeability (or holes) of the vesicles to the enzyme.

The total amount of bound labelled Con A was about 30% of the incubated amount for R.S.O. and less than 2% for I.O. This clear cut result shows that in contrast to the neuraminidase, the large mol. wt Con A (50 000 daltons) is not able to penetrate the I.O. vesicles even after 30 min incubation.

From 10 different plasma membrane isolations, 20 separations of vesicles populations were performed:

Table 1
Properties of three different subpopulations of MOPC 173 plasma membranes

Material	Proteins (mg)	Sialic acid (mmol/mg protein		Bound Con A (% incubated amount)	Specific activity (μmol P_i liberated/hr/mg protein	
		Total amount	Released by neuraminidase		5′-nucleotidase	($Na^+ + K^+$) ATPase
Purified plasma membranes	100	104 ± 7	65 ± 5	n.d.	15	6.3
IO. vesicles	40 ± 5	127 ± 10	0 ± 5	< 2%	21	9
R.S.O. vesicles	40 ± 5	121 ± 8	110 ± 8	# 30%	8.5	3.6

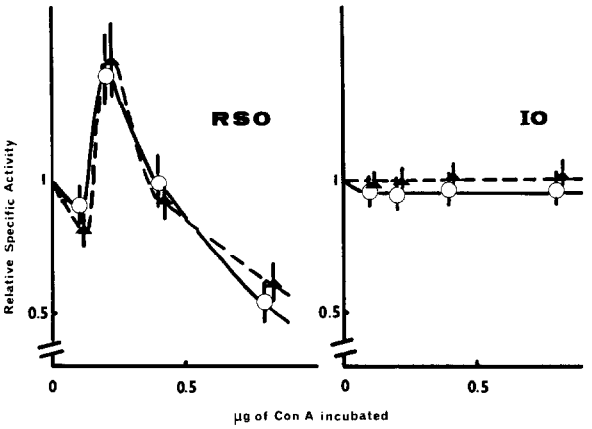

Fig.1. Modulation of the 5′-nucleotidase (o–o–o) and (Na^+ + K^+) stimulated Mg^{++} ATPase (▲–▲–▲) activities by adding various amounts of Con A to R.S.O. (left) or I.O. (right) vesicles. The relative specific activity is the ratio between the specific activity in presence of Con A and the specific activity in absence of Con A.

the specific activities of 5′-nucleotidase and (Na^+ + K^+) stimulated Mg^{++} ATPase, expressed in µmol P_i/hr/mg of protein were for a given experiment respectively 21 and 9 for I.O. vesicles and 8.5 and 3.6 for R.S.O. vesicles. In all instances, total activities recovered in both populations represented about 80% or the total activities found in the original purified membranes. From these results we can deduce that R.S.O. are at least permeable to ATP and AMP.

To 40 µg of plasma membrane proteins increasing amounts of Con A from 0.1 to 1 µg were added; the mixtures were then incubated for 30 min at 37°C. Fig.1 illustrates the dose-dependant modulation of both enzymatic activities under study when Con A is added either to I.O. or to R.S.O. vesicles. Whereas I.O. vesicles showed no modification of the enzymatic activities whatever was the dose of Con A, R.S.O. expressed a biphasic reaction for both enzymes: from 0.1 to 0.4 µg of Con A, the 5′-nucleotidase and the (Na^+ + K^+) stimulated Mg^{++} ATPase increased from 100 to 130–160%, while from 0.5 to 1 µg of Con A the activity came down to 40%. We would like to stress the point that with some batches of Con A, with the same amount of lectin, increase reached 200%. The calculations showed that 10^{12} molecules stimulated activities whereas $5 \cdot 10^{12}$ and over inhibited them.

Fig.2 illustrates the dose dependant modulation of the same enzymatic activities when the peptidoglycan is incubated with either I.O. or R.S.O. vesicles, the procedure being the same as above. In this case, R.S.O. were not reactive at all i.e. both enzymatic activities stayed at the same level whatever was the dose of adjuvant added, between 10^{-12} to 10^{-5} µg/ml. In contrast I.O. vesicles showed a biphasic reaction for both enzymes: increase to 120–150% was observed between 10^{-9} and 10^{-7} µg/ml where inhibition occurred between 10^{-6} to 10^{-3} µg/ml. The calculations showed that only $2 \cdot 10^5$–$2 \cdot 10^7$ molecules stimulated whereas $2 \cdot 10^8$–$2 \cdot 10^{10}$ inhibited the activities.

4. Discussion

From these results we can conclude that our procedure allowed us to separate I.O. and R.S.O. vesicles characterized mainly by the fact that glycoproteins are exposed only in R.S.O. Thus it is understandable that Con A is acting only on R.S.O. and not at all on I.O. R.S.O. are permeable to neuraminidase to AMP and ATP not to Con A which would mean that vesicles are not closed but have only small holes. It is important to notice that both enzymes are modulated with the same dose curve which lead us to hypothetize that either Con A has a non specific effect

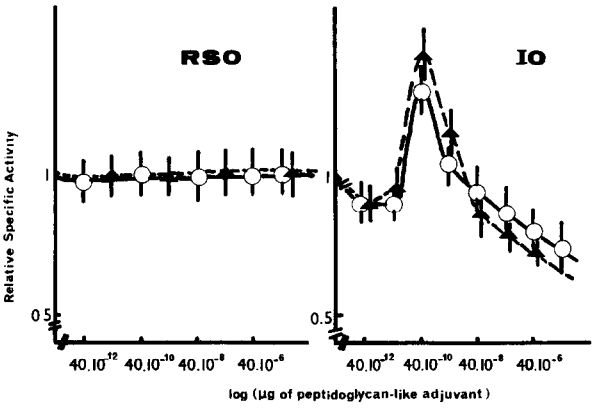

Fig.2. Modulation of the 5′-nucleotidase (o–o–o) and (Na^+ + K^+) stimulated Mg^{++} ATPase (▲–▲–▲) activities by adding various amounts of acetylated peptidoglycan-like adjuvant to R.S.O. (left) or I.O. (right) vesicles.

i.e. by modifying the membrane structure, or that, as both enzymes could be glycoproteins [28,29] Con A has a direct effect on the enzymatic activities. Con A is known to induce patching and sometimes capping [30,31] and this has been shown to be true in the case of different variants of our culture cell lines [32]. We would favor the idea that depending upon the percentage of the binding sites of the surface membrane bound by the lectin, structural changes differ so that the enzyme activities would be enhanced or inhibited in a non specific way. This figure would mimic antigenic stimulation or inducement of tolerance depending upon the percentage of specific receptors bound [33,34]. It is important to emphasize the fact that in contrast to Con A the acetylated peptidoglycanlike adjuvant is active on I.O. and not on R.S.O. As this adjuvant was extracted from a lipid rich fraction, it can be assumed that the molecule should reach the lipidic moiety of the cell membrane in order to be active and that I.O. vesicles allow easily the molecule to penetrate the membrane whereas R.S.O. do not. We have to study where is sitting the molecule into the membrane for an understanding of its activity.

For both adjuvants we observed with low and high doses enhancement and inhibition respectively for both 5′-nucleotidase and $(Na^+ + K^+)$ stimulated Mg^{++} ATPase. We could hypothetize that adjuvants could be stimulating or inhibiting depending upon the dose used. The stimulating effect is well documented as well at the host or the cell level [2] and inhibition has also been described at the host [35,36] and the cell level [6,37]. The membrane vesicles seem to be an extremely sensitive test as it requires much less active product to test its activity: 10^{-7} g of Con A is enough compared to 10^{-5}–10^{-6} g in tissue culture [6,37]; 10^{-16} g of peptidoglycan-like substance was revealed in our system compared to 10^{-8}–10^{-9} g on cell macrophages [38,39]. In this last example, we do not know how so low doses are still active.

The results presented here seem to indicate that our procedure could be used to screen adjuvants. It seems that Con A and the peptidoglycan while acting at two different levels of the plasma membrane, induce both a biphasic modulation of 5′-nucleotidase and of the $(Na^+ + K^+)$ stimulated Mg^{++} ATPase: this result is in favor of an indirect effect of the adjuvants on the enzymatic activities.

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