BIOCHEMICAL JOURNAL LETTERS

A comparison of iodinated and technetium-labelled zymosan for measurement of particle binding and internalization by macrophages

Many measurements of phagocytosis are undermined by a failure to distinguish cell surface binding and genuine internalization (see review by Kavet & Brain, 1980). Common methods for dissociating surface-bound particles using enzymes, chelators or polyanions do not remove zymosan (a yeast cell wall particle) from the macrophage surface (Kavet & Brain, 1980; J. L. Bodmer & R. T. Dean, unpublished work).

Recently in a very careful paper a method using radioactive ^{99m}Tc -labelled zymosan (^{99m}Tc -zymosan) to distinguish binding and internalization by alveolar macrophages has been described (Besterman *et al.*, 1982). After exposure of macrophages to ^{99m}Tc -zymosan a substantial proportion of the cell-associated radioactivity can be dissociated from the cell by competition with large amounts of unlabelled zymosan. This fraction is not accounted for by inadequate washing, zymosan contained in detached cells, or exocytosis, so the fraction has been described as reversibly bound to the cell surface. Thus the authors have concluded that dissociation of surface-bound ^{99m}Tc -zymosan occurs. However we have found using this method with mouse peritoneal macrophages that it is merely the technetium that is displaced, not the whole particle; and that label from iodinated zymosan cannot be dissociated. Since this is a significant technical distinction, which could lead to misinterpretation, we present here our data to that effect.

We have measured both binding and phagocytosis of, on the one hand, ^{125}I -labelled zymosan, and on the other, ^{99m}Tc -labelled zymosan (prepared as described by Besterman *et al.*, 1982, except that no serum was added to the preparation). Uptake (the sum of surface binding and phagocytic internalization) was followed at 37°C , and surface binding was measured in two ways: either at 4°C or at 37°C in the presence of $10\mu\text{g}$ of Cytochalasin B/ml. Experiments at 4°C were done using pre-gassed and cooled medium. Binding was measured by aspirating the medium, washing the cell layer four times with ice-cold phosphate-buffered saline and

then lysing the cells with phosphate-buffered saline containing 0.1% Triton X-100 and measuring its associated radioactivity. Using these methods we found that both labelled forms of zymosan gave similar estimates of uptake (approx. $185\mu\text{g}$ uptake/mg of cell protein in 1.5 h).

For measurements of dissociation after the washing step 2 ml of zymosan suspension (1 mg/ml) was added to the plates which were then re-incubated at 4°C for various times. The dissociation fraction was collected and counted, as was the cell fraction. For these measurements, besides the two singly-labelled preparations we also used a doubly-modified preparation of zymosan that had been treated with non-radioactive technetium (^{99}Tc) and then radioactively labelled with iodine. The same method for following dissociation was used for ^{125}I -zymosan, ^{99m}Tc -zymosan and the doubly modified ($^{99}\text{Tc} + ^{125}\text{I}$)-zymosan. Experiments using ^{99m}Tc -zymosan labelled with non-radioactive iodine could not be performed satisfactorily because of the technical restrictions of our iodination method using Iodogen.

The representative results in Fig. 1 show that ^{99m}Tc radioactivity does dissociate from the cells while there is little dissociation of ^{125}I radioactivity. There are two main possible explanations for this difference; one is that the dissociation is due to exchange of ^{99m}Tc between the bound, and the free, unlabelled zymosan, while the other is that zymosan itself is exchanging, carrying technetium, as assumed by Besterman *et al.* (1982). The fact that the zymosan labelled with both non-radioactive ^{99}Tc and iodine does not dissociate (Fig. 1) supports the former possibility, that ^{99m}Tc exchanges between bound and free zymosan.

Our data do not necessarily undermine the quantification of compartments suggested by Besterman *et al.* (1982). Their quantification requires only that the dissociated radioactivity comprises all the surface-bound radioactivity; it does not depend on the form in which the radioactivity is released or on dissociation of zymosan itself. Our measurements of compartments agree well with theirs; after 1.5 h exposure to zymosan at 37°C both labels gave an estimate of the fraction of zymosan on the cell surface of 0.2–0.3. Thus it seems that the estimates of compartments obtained by Besterman *et al.* (1982) are correct; but that surface technetium can dissociate from the cells without release of the

Fig. 1. Dissociation of $^{99\text{m}}\text{Tc}$ but not zymosan from macrophage surfaces

Mouse peritoneal macrophages were collected as described previously (Dean *et al.*, 1979) and maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated pig serum, 100 i.u. of penicillin/ml and 100 μg of streptomycin/ml. At the start of the experiments the cultures were washed four times in phosphate-buffered saline and reincubated for particle uptake for 1.5 h in fresh medium containing zymosan suspensions as appropriate. The total zymosan concentration in all cultures was 50 $\mu\text{g}/\text{ml}$. The cultures were then treated as described in the text for measurement of dissociation. Data are means $\pm$ S.D. ($n = 3$) and are representative of several experiments. $\circ$, ^{125}I -zymosan; $\triangle$, $^{99\text{m}}\text{Tc}$ -zymosan; $\square$, ($^{125}\text{I} + ^{99\text{m}}\text{Tc}$)-zymosan.

zymosan particles. As a corollary, in our particular conditions, intracellular technetium must be satisfactorily retained by the cells, perhaps because when released from zymosan the molecules become attached to cellular proteins. It is important to realize that technetium release does not necessarily indicate zymosan exchange, and thus that measurement of surface compartments of zymosan, using the dissociation induced by large amounts of unlabelled zymosan, is not generally feasible when the zymosan has been covalently labelled, as in our experiments. It should also be pointed out that we have used adherent macrophages, though Besterman *et al.* (1982), and several other groups, have employed suspensions of cells. Both the state (adherent versus suspended) and nature (alveolar versus peritoneal) of the cells may have some influence on the behaviour of surface-bound particles.

The present data thus show that surface-bound zymosan particles do not dissociate when exposed to large quantities of zymosan in the extracellular fluid. They therefore emphasize the need for a method for removing cell-surface-bound zymosan without killing or grossly modifying the cells, so as to be able to study more specifically the effect of surface-bound zymosan. Recent evidence (Sung *et al.*, 1983;

Bodmer & Dean, 1983) indicates that a significant part of the zymosan binding is at the mannose-glycoprotein receptor (Stahl *et al.*, 1978) of macrophages; this implies that dissociation may be achieved by exposure to low pH.

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Reply

We have read with great interest the preceding BJ Letter by Bodmer & Dean (1983). It is, indeed, critical to an understanding of phagocytosis that both particle binding and internalization be analysed quantitatively, and it is unfortunate that so few data are available in the literature in this regard. As well, careful design and interpretation of experiments which discriminate the two processes from one another are essential.

Bodmer & Dean (1983) present experiments which suggest that technetium, bound to zymosan on macrophage surfaces, can exchange with free, unlabelled zymosan added as part of wash (dissociation) protocols; however, there are experiments which remain to be done to confirm the interpretation of the results described in their letter. It would be important to show that iodine-labelled zymosan binds to macrophages by the same mechanism as does technetium-labelled zymosan. In this regard, it would be instructive to label the same zymosan preparation with both radioactive technetium and radioactive iodine and then show that