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BINDING OF A SPIN LABEL ANALOGUE OF COMPOUND 48/80 TO RAT PERITONEAL MAST CELLS: CORRELATION OF BINDING PROPERTIES WITH SURFACE TOPOGRAPHY

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Abstract—A spin label analogue of compound 48/80 has been synthesized and its binding to purified rat peritoneal mast cells has been studied by electron spin resonance spectroscopy. The spin label analogue (SL-48/80) had almost identical biological activity to unlabeled compound 48/80. SL-48/80 was used to estimate the number of binding sites per cell on normal mast cells (7.25×10^{10}), on mast cells deactivated by sodium azide and 2-deoxyglucose or by heating to 46° for 30 min (1×10^{10}) and cells from animals actively-sensitized to ovalbumin (5.2×10^{10}). SL-48/80 was also shown to bind to isolated mast cell granules. Differences in the binding properties of mast cells after the different treatments are related to their surface topography as seen by scanning electron microscopy, and the contribution of the granules to the number of binding sites is discussed.

The pharmacological properties of compound 48/80, a polymer formed by reacting equimolar proportions of *p*-methoxy-*N*-methylphenylethylamine and formaldehyde [1], are well established [2, 3]. 48/80 has been shown to bind to receptors on the external membranes of mast cells [4], resulting in degranulation and the release of histamine. This process is energy and calcium-dependent and can be suppressed by metabolic inhibitors, e.g. sodium azide and 2-deoxyglucose [3].

Using ESR† spectroscopy, Ortner and Chignell demonstrated that spin-labeled 48/80 could bind reversibly both to mouse mastocytoma cells and to rat mast cells [5]. They concluded that the binding properties of spin-labeled 48/80 to mast cell membranes were consistent with the binding sites being associated with the proteins of the external membrane [6]. These latter studies were concerned mainly with mastocytoma cells rather than with purified mast cell preparations.

In this paper we describe the use of ESR spectroscopy to study the binding of a spin-labeled analogue of 48/80 (different from that employed by Ortner and Chignell [4, 5]) to purified rat mast cells from both normal and actively-sensitized animals and the effect of azide ions and 2-deoxyglucose on the binding. By the use of Scatchard plots, the technique has been used to estimate the number of binding sites for the spin label per mast cell as well as their binding constants. Differences in the binding properties of the spin label under the different conditions employed are correlated with the mast cell surface topography as seen by scanning electron microscopy.

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† Abbreviations: ESR, electron spin resonance; SL-48/80, spin label analogue of compound 48/80; BSA, bovine serum albumin; BSSA, buffered salt solution containing albumin.

MATERIALS AND METHODS

Compound 48/80, 2-deoxyglucose, *p*-methoxyphenylethylamine and sodium cyanoborohydride were obtained from Sigma Chemical Co. 4-Oxo-2,2,6,6-tetramethylpiperidinoxy was from Aldrich Chemical Co. All other chemicals were the best grades available.

Isolation and purification of rat peritoneal mast cells. Mast cells were obtained from male Colworth Wistar-derived rats (approximately 200 g body weight) as described previously [7]. A buffered salt solution (BSSA) of the following composition was used: NaCl, 139 mM; KCl, 2.4 mM; $CaCl_2$, 0.8 mM; Sorensen phosphate buffer, 6.7 mM (pH 6.8); dextrose, 5.6 mM; bovine serum albumin, 0.1% w/v. The medium included heparin (5 units/ml) for harvesting only.

The mast cell preparations (combined from 8–10 rats and containing 5–10% mast cells) were purified by a BSA density gradient method [8]. The mast cell fraction was washed twice to remove excess albumin, resuspended in BSSA, counted [7] and diluted in BSSA to give 1.6×10^6 mast cells/ml. After purification, the mast cells were 94.4 ± 0.5% SEM pure. Recovery was >70%. Plastic laboratory ware was used throughout the preparation.

Granules were prepared by sonication of purified mast cells followed by centrifugation according to the method of Ludowyke *et al.* [9].

Sensitization of rats. Rats of approximately 100 g body weight were sensitized using a procedure similar to that described by Jasani and Stanworth [10]. The animals were given a single subcutaneous injection of *B. pertussis* vaccine (1×10^{10} organisms) and 100 μg ovalbumin in 0.2 ml saline. Mast cells were harvested and purified as described above, 21–28 days later. A net release of 15–20% of the total histamine content was evoked when the cells were challenged with specific antigen (50–500 μg/ml).

Deactivation of mast cells. Mast cells were deactivated either by preincubation for 40 min (37°) in BSSA containing 10^{-2} M sodium azide with 2-deoxyglucose substituting for glucose (6.1 mM) or by heating the cells to 46° for 30 min followed by cooling to 37° for 30 min [3].

Synthesis of NOR 48/80. Equimolar proportions of *p*-methoxyphenylethylamine and formaldehyde in 6 M HCl were heated in a stoppered tube at 100° for 4 hr [1]. The mixture was evaporated down under reduced pressure and chromatographed on a column of Sephadex G-25 superfine in 0.03 M acetic acid. The fractions corresponding in elution volume to commercial 48/80 were bulked and freeze dried.

*Synthesis of SL-*48/80. Spin-labelled 48/80 was prepared by addition of nitroxide ketone derivative to NOR 48/80 (a primary amine analogue of compound 48/80) and reduction of the resulting Schiff's base by sodium cyanoborohydride [11].

NOR 48/80 (10 mg) and 4-oxo-2,2,6,6-tetramethylpiperidinoxy (1.7 mg) were dissolved in methanol (2 ml) with the addition of triethylamine (1 drop). Sodium cyanoborohydride (5 mg) was added to this solution and the mixture was stirred for 2 hr and then left to stand overnight. The solvent was then blown off with N_2 and the residue dissolved in 1 M acetic acid. The mixture was chromatographed on Sephadex G-25 superfine and the fractions containing SL-48/80 were identified by UV and ESR spectroscopy. The extent of labelling was determined as 0.65 nitroxide groups per hexameric unit of NOR 48/80 (the hexamer has been shown to be the most active oligomer of compound 48/80 [12]).

ESR spectroscopy. ESR spectra were obtained on a Varian E-4 X-band spectrometer fitted with an E257–9 variable temperature accessory, using a quartz aqueous sample cell. Sample temperature was monitored using a copper–constantan thermocouple.

*Binding of SL-*48/80 *to mast cells.* Stock solutions of SL-48/80 (about 10 mg/ml) were added using a microsyringe to 200 μl aliquots of cells (1.6×10^6 cells/ml) in BSSA or to 200 μl aliquots of BSSA alone as controls. Concentrations of SL-48/80 solutions were determined from their UV absorbance at 277 nm

$$E^{1\%}_{277} = 65.3 .$$

The addition of SL-48/80 to a suspension of mast cells resulted in a reduction in the intensity of the nitroxide ESR signal compared with that obtained when BSSA alone was used. This reduction of intensity was accompanied by the appearance of a broad signal of much lower amplitude arising from SL-48/80 bound to the mast cells.

Calibration plots were constructed of the relationship between the high-field peak height of the SL-48/80 ESR spectrum and concentration, run under identical conditions of modulation amplitude, microwave power, etc., for each experiment. These plots were always linear over the concentration range employed.

Concentrations of free and bound SL-48/80 were calculated from the difference in intensity of the high-field line of the ESR spectrum between mast cell-containing samples and control values derived from the calibration plot. The high-field line is used for these measurements as it is the line least affected in intensity by the presence of the broad ('bound') ESR signal.

Histamine release assay. An automated fluorimetric assay based on the procedure described by Siraganian [13] was used for the determination of histamine. This procedure has been described in detail elsewhere [7].

Scanning electron microscopy (SEM). Aliquots (200–400 μl) of suspensions of purified mast cells in BSSA ($2–4 \times 10^5$ cells) were seeded on to 22 mm dia. glass or 25 mm dia. plastic (Thermanox, No. 1½) coverslips in a Petri dish. The cells were allowed to sediment on to the coverslips by gravity for periods of up to 60 min by incubating at 37° in an atmosphere of 95% air + 5% carbon dioxide and 100% humidity.

Coverslips with monolayers of adherent cells were:

(i) rinsed and fixed in freshly-prepared 2% glutaraldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min at room temperature, followed by washing in buffer prior to processing for SEM, or

(ii) incubated further at 37° for 30 min with various concentrations of 48/80 in BSSA, followed by fixation as above.

After glutaraldehyde fixation and washing, the cells were dehydrated through successive ethanol–water mixtures (50% through 90% and 100% × 3) for 15 min each. The samples were then critical point dried via carbon dioxide in a Polaron E3000 critical point bomb. The coverslips were mounted on brass specimen stubs using colloidal silver and coated with 150 Å of gold in a Polaron E5100 'cool' Splutter Coating Unit. The cells were examined in a JEOL JSM-35X scanning electron microscope at 25 kV accelerating voltage and micrographs taken with Polaroid type 55 P/N film.

RESULTS

Spin-labelled 48/80 *(SL-*48/80)

The presumed chemical structure of SL-48/80 is shown in Fig. 1; the exact position of the nitroxide radical on the polymer is not known. NOR 48/80 was used for the synthesis because the presence of the *N*-methyl groups in 48/80 was found to inhibit the labelling reaction. NOR 48/80 has been reported to be 83% as potent as compound 48/80 itself [14].

The histamine release figures for SL-48/80 along with those for a commercial sample of 48/80 are shown in Table 1. The biological activity of the spin-labelled compound is clearly very similar to that of compound 48/80.

*Binding of SL-*48/80 *to mast cells*

ESR spectra of SL-48/80 alone in BSSA and in the presence of mast cells are shown in Fig. 2. Spin label molecules tumbling freely in solution give rise to narrow ESR signals of high intensity; immobilization of the spin labels leads to broadening of the signals accompanied by a loss of intensity. In Figs 2b and 2c the broad signal arising from the bound spin probe can be seen clearly together with the three narrow lines from the free spin probe.

ESR spectra of SL-48/80 in the presence of mast cells at room temperature were initially run at 5 min intervals up to 30 min. Since no changes in the

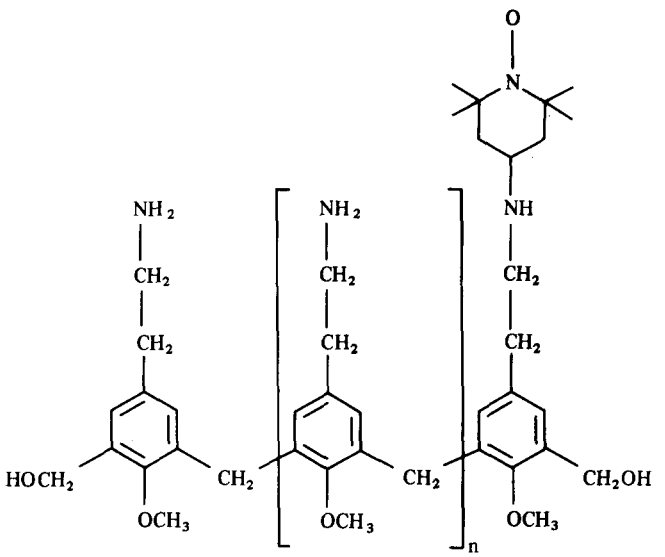

Fig. 1. SL-48/80.

ESR spectra were observed after the first 5 min, measurements for the construction of Scatchard plots were taken from spectra run 10 min after mixing.

Ortner and Chignell [5] reported that their spin label analogue of 48/80 was rapidly metabolized by purified rat peritoneal mast cells, resulting in the disappearance of the ESR signal. In order to test for this, samples from a mixture of SL-48/80 with mast cells in BSSA were taken at 5 min intervals, denatured with a mixture of sodium dodecyl sulphate and sodium hydroxide and their ESR spectra monitored. No evidence of metabolism of the spin label described in this paper was found over 30 min.

It has been suggested [15] that at concentrations above 100 μg/ml, 48/80 can behave as a detergent. Association of SL-48/80 molecules into micelle-like structures would be expected to give rise to line broadening and hence a reduction in signal amplitude due either to reduced mobility of the probe or to nitroxide spin exchange. The fact that plots of SL-48/80 peak height vs concentration were linear (up to 300 μg/ml) shows that line broadening from this source was not in evidence.

Addition of compound 48/80 to samples of mast cells to which SL-48/80 had been added resulted in an increase in the amplitude of the free label ESR signal, arising from displacement of bound SL-48/80 by unlabelled material. This showed that SL-48/80 and compound 48/80 were competing for the same binding sites on the mast cell, and that the binding of SL-48/80 was reversible.

Addition of SL-48/80 to unpurified peritoneal cell suspensions of the same total cell count (1.6×10^6/ml), resulted in a very much lower extent of binding of the spin label than was observed with purified mast cells, thus demonstrating the preferential binding of SL-48/80 for mast cells.

A typical Scatchard plot [16] for the binding of SL-48/80 to mast cells is shown in Fig. 3. The number

Table 1. Histamine release from mast cells by 48/80 and SL-48/80

Concentration of activator (ng/ml)	% release (net) by 48/80	% release (net) by SL-48/80
200	90.1	90.2
150	88.5	90.4
100	89.4	91.3
50	79.0	86.7
25	58.3	61.6
10	20.5	22.3
5	3.5	5.1

10^4 cells/ml; total histamine, 252 ng/ml; spontaneous release, 3.43%.

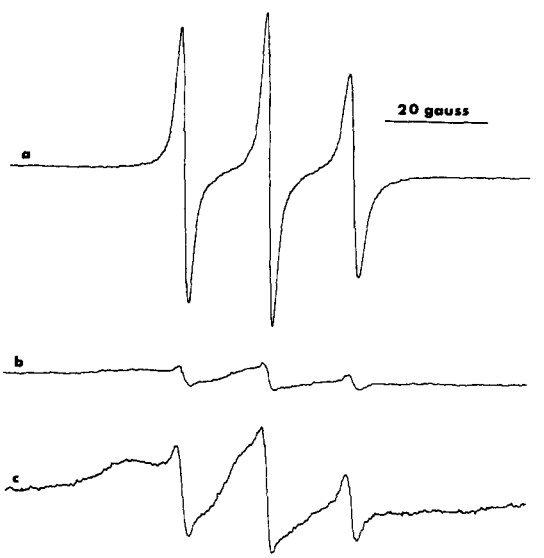

Fig. 2. ESR spectra of SL-48/80 (a) 312 μg/ml in BSSA, (b) 156 μg/ml + 1.6×10^6/ml rat peritoneal mast cells in BSSA, (c) as (b) run with 2 × modulation amplitude and 2.5 × receiver gain.

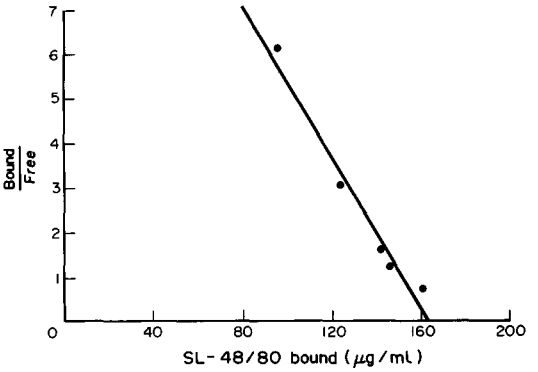

Fig. 3. Scatchard plot of the binding of SL-48/80 to rat peritoneal mast cells (1.6×10^6/ml) in BSSA.

of binding sites for SL-48/80 per mast cell (abscissa) and the binding constant (ordinate) were calculated from the intercepts of the two axes. The intercepts on the two axes were calculated by linear regression analysis. The parameters for the binding of SL-48/80 to normal mast cells in the presence and absence of sodium azide and 2-deoxyglucose, and to mast cells from animals sensitized to ovalbumin are shown in Table 2. The saturation binding figure of 7.25×10^{10} SL-48/80 molecules per mast cell compares well with the value of 6×10^{10} obtained by Morrison *et al.* [3] using ^{131}I-labelled 48/80.

It has been shown [3] that incubating mast cells for 40 min in medium containing 10^{-2} M sodium azide with 2-deoxyglucose substituted for glucose or heating suspensions of mast cells to 46° for 10 min inhibits the cells in such a way that they are unable to respond to a non-cytotoxic histamine releasing stimulus; hence there is no degranulation or exposure of the granules to the extracellular medium.

In the presence of deactivating levels of sodium azide and 2-deoxyglucose, the binding of SL-48/80 to mast cells was reduced significantly (see Table 2). We were unable to construct Scatchard plots giving a clear dependence of binding on added SL-48/80 from the experimental values in the presence of azide and 2-deoxyglucose, therefore only the saturation binding levels of SL-48/80 are recorded for these experiments. Addition of SL-48/80 to mast cells inactivated by heat treatment resulted in an identical saturation binding level (1×10^{10} molecules/cell) to those obtained with azide and 2-deoxyglucose.

Mast cells from animals sensitized to ovalbumin showed a small but significant reduction in the number of binding sites for SL-48/80 and an apparent increase in binding constant compared with mast cells from normal animals.

One explanation which has been proposed for the difference in the binding levels of 48/80 between normal and deactivated mast cells is that in the former case, the activator can bind extensively to the granules [3]. In order to test this hypothesis, a preparation of mast cell granules was made. SL-48/80 was found to bind extensively to mast cell granules. The addition of SL-48/80 also resulted in an increase in the turbidity of the granule preparation. Examination under the microscope revealed that aggregation of the granules had taken place. There is considerable uncertainty about the exact recovery of granules extracted from mast cells (due to the difficulty in counting the isolated granules); however, based on an estimated recovery of 25%, binding of SL-48/80 to granules accounted for between 4 and 5×10^{10} binding sites per mast cell. This preliminary observation suggests that at least a large part of the binding of SL-48/80 to mast cells can be accounted for by binding to granules.

Scanning electron microscopy (SEM)

Examination by SEM showed the normal purified mast cell to be spherical or slightly flattened when in contact with the underlying coverslip. Adherence was by short, thin, tapering processes. The surface of the cell was covered by numerous protruding, branching folds (Fig. 4a) which were equivalent to the 'microvilli' observed in vertical-section transmission electron microscopy (not shown). No differences in the surface morphologies of purified and unpurified mast cells were noted. SEM examination of mast cells adherent to coverslips and treated with BSSA containing sodium azide and 2-deoxyglucose showed a complete loss of external folds from the membrane to give contourless surface features (Fig. 4b). Heat-deactivated mast cells also exhibited the same lack of external membrane features. Higher magnification, however (Fig. 4c), showed that the surfaces of these cells were still quite rough and nodular in appearance.

Table 2. Binding Parameters of SL-48/80 to mast cells

	No. of binding sites/cell	Binding constant (M^{-1})
Normal cells	$7.25 \pm 0.48 \times 10^{10}$ (N = 8)	$8.68 \pm 0.66 \times 10^4$ (N = 7)
Normal cells + NaN_3 (10 mM) and 2-deoxyglucose (6.1 mM)	$1.03 \pm 0.27 \times 10^{10}$ (N = 4) P < 0.001	N.D.
Sensitized cells	$5.20 \pm 0.58 \times 10^{10}$ (N = 4) P < 0.05	$1.54 \pm 0.19 \times 10^5$ (N = 3) P < 0.01

The number of binding sites/cell and binding constants were calculated using a molecular weight of 1000 for the hexameric unit of SL-48/80. The values were subjected to a test for normality (Filliben test) and outlying values were eliminated by the Grubb procedure. Error values quoted are standard error of mean (S.E.M.), P values from two-tailed Student's *t*-test.

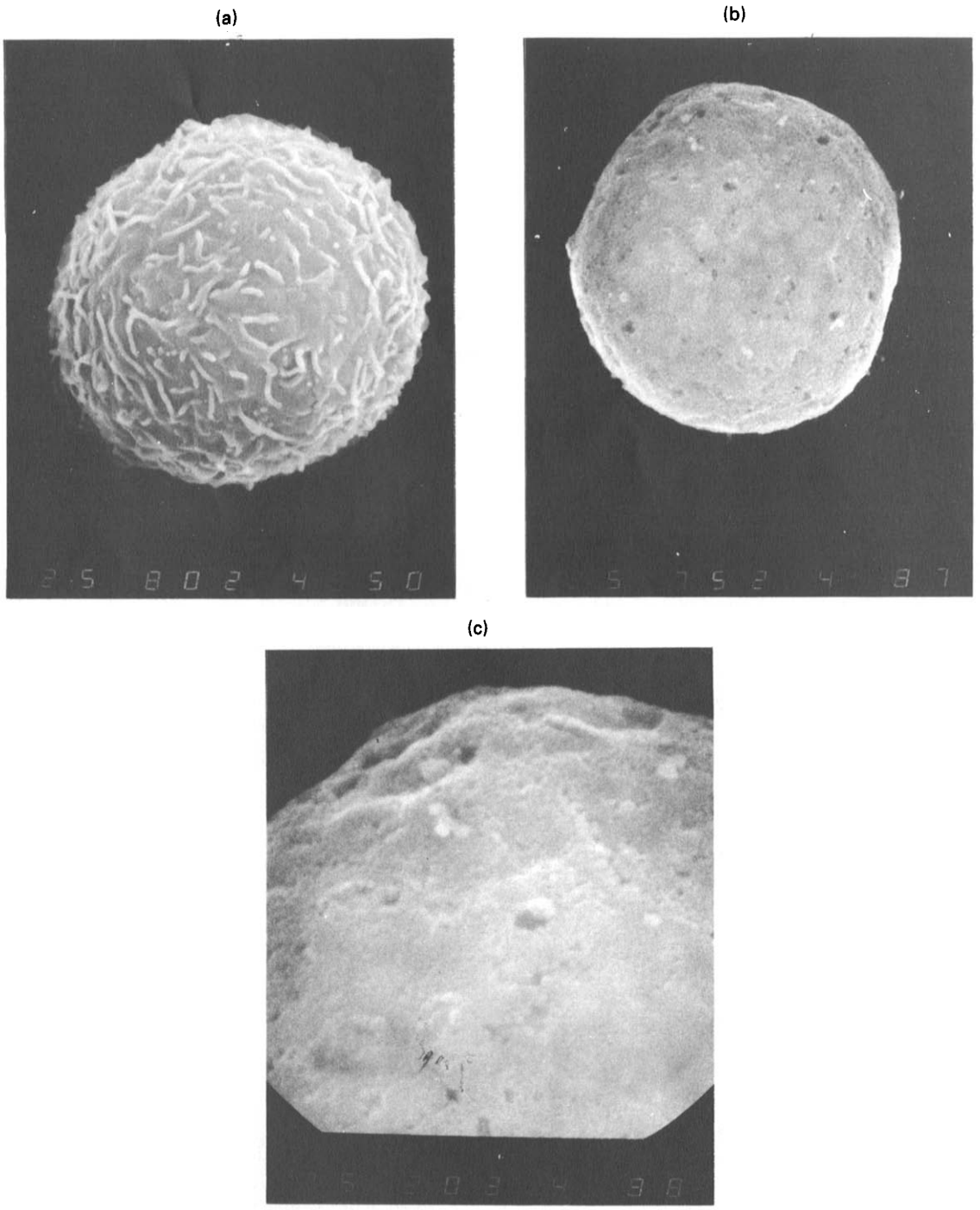

Fig. 4. Scanning electron micrographs of rat peritoneal mast cells after treatment in (a) BSSA (8000 × magnification), (b) BSSA + NaN_3 (10 mM) + 2-deoxyglucose (6.1 mM) (7500 × magnification) and (c) as (b) (20,000 × magnification).

An increase in surface fold length was noted in purified mast cells taken from sensitized animals.

DISCUSSION

It has been shown in the present work and elsewhere [3] that the binding of 48/80 or its spin label analogue to mast cells is considerably reduced when the cells are deactivated by deoxyglucose and azide or by heating to 46° and also that these differences in binding are accompanied by changes in the surface topography of the cells. The question arises as to what extent the differences in the number of binding sites can be correlated with the surface topography.

The surface area of a mast cell (diameter 12 μm) is 4.8×10^8 nm^2. If we assume that a molecule of SL-48/80 would occupy between 1 and 4 nm^2, the number of binding sites which would be accom-

modated on the surface of a smooth sphere of that diameter is between 1.2×10^8 and 4.8×10^8. Clearly, the smooth sphere model cannot account for the 1×10^{10} SL-48/80 molecules bound to deactivated mast cells; the surface of the deactivated cell—apparently relatively smooth as seen by SEM—must contain very many ridges and pockets to accommodate this number of binding sites.

One possibility is that some of the SL-48/80 molecules may penetrate to binding sites located within the membrane of the mast cell. ESR evidence from experiments on mastocytoma cells [5] shows that spin-labelled 48/80 molecules can bind at sites away from contact with ferricyanide ions in the aqueous medium, suggesting that some of the binding sites are located deep within the membrane. Many binding sites on mast cells are known to be surface-located since NOR 48/80 attached to Sepharose beads has been shown to bind to the exterior of the cells [4].

The difference in the number of binding sites for SL-48/80 between normal and deactivated mast cells may be attributed partly to the fact that normal cells exhibit considerably more folding of their surface than deactivated cells. The release of granules also contributes extensively to the binding of SL-48/80; their exact contribution to the binding may be known better when more accurate estimates both of the recovery of granules from mast cells and the number of granules per mast cell are available. Numbers of granules per mast cell range from between 250 and 500 [17] to over 1000 [18]. In addition to the contribution from binding to the granules themselves, their exit from the mast cell makes available the perigranular membrane which also has the potential to bind a large number of 48/80 molecules [19]. Establishing any correlation between binding sites and extent of folding of the cell surface may ultimately depend on finding a means of deactivating the mast cell whilst maintaining its normal topography.

We can offer two possible explanations for the lower number of binding sites found on mast cells from rats sensitized to ovalbumin. One possiblity is that some of the binding sites for 48/80 on the cell membrane could be blocked due to the binding of specific IGE to the cell surface IGE-receptors [20]. Since binding to the cell membranes accounts for a minority part of the total binding to normal cells, almost all of the cell surface sites would have to be blocked by IgE to account for the reduction in binding in cells from sensitized animals compared with normal ones.

The work of Hertel *et al.* [21] using electron microscopy offers a more likely explanation. They suggest that a new population of mast cells arises after sensitization. Although larger than normal mast cells, the new cells contain a considerably lower number of granules, which might account for the larger part of the difference in binding of SL-48/80 between normal and sensitized cells.

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