

SURFACE EXPRESSION OF FUNCTIONAL IgE BINDING PROTEIN, AN ENDOGENOUS LECTIN, ON MAST CELLS AND MACROPHAGES¹

LUCIANO G. FRIGERI² AND FU-TONG LIU

From the Department of Molecular and Experimental Medicine, Research Institute of Scripps Clinic, La Jolla, CA 92037

IgE-binding protein (εBP) is a galactoside-specific lectin containing an S-type carbohydrate-recognition domain. It was originally identified in rat basophilic leukemia cells and is now known to be identical to a macrophage surface Ag, Mac-2, and lectins designated as CBP 35/L-34/RL-29. It has also been related to a nonintegrin laminin-binding protein isolated from mouse macrophages. In this report we have shown the following: εBP is present in variable amounts in several mast cell lines, and the surface expression of εBP in these cell lines is quite variable and does not correlate with the total amount of εBP in the cell. εBP is displayed on the cell surface in a manner that is reversible by lactose, most likely through attachment to yet unidentified glycoconjugates. The putative εBP binding sites on the cell surface can be readily demonstrated by using radiolabeled εBP, and the sites are present in comparable amounts in various cell lines. Expression of εBP on the cell surface can be regulated; the most notable example is the upregulation of surface εBP on RBL cells activated through the high-affinity IgE receptor by IgE immune complexes. Cell-surface εBP is functional as measured by its ability to promote adhesion of trypsinized rabbit erythrocytes to mast cells and macrophages. On the basis of these results and reported properties of related lectins, we propose that the lectin represented by εBP is a new class of cell-adhesion protein.

εBP³ was originally identified in rat basophilic leukemia cells by virtue of its affinity for IgE (1–4). Subsequently, it was found to be a β-galactoside-binding lectin variously designated as CBP 35, RL-29 (HL-29), L-34, and Mac-2 (5–8). εBP has the following characteristics: 1) It has a *M_r* of 29,000 to 35,000, with highly conserved amino acid sequence among different species. 2) It is composed of two structural domains; the amino-terminal domain consists of tandem repeats, and the carboxyl-terminal por-

tion contains an S-type carbohydrate recognition domain (9). 3) It lacks an apparent transmembrane domain and signal sequence, although it is found in the extracellular space. 4) It is expressed on the cell surface and in the cytoplasm and nucleus. 5) It has a wide tissue distribution and is expressed by various cell types.

The function of εBP has not been established. Studies with CBP 35 (10, 11) suggested that the lectin may be a component of a growth regulatory system. The protein was expressed at an elevated level in proliferating cells and was copurified with heterogeneous nuclear ribonucleoprotein complexes. Studies with L-34 (12) suggested that the lectin may be a marker for neoplastic transformation and metastatic potential of melanoma cells. Mac-2 is expressed on maturation of macrophage precursors and in macrophages elicited by inflammatory stimuli (13). Finally, a nonintegrin laminin-binding protein has been found to be identical to Mac-2/CBP 35/εBP (14), suggesting that the lectin may be involved in adhesion of macrophages or mast cells to basement membranes.

To better understand the function of εBP, we decided to focus on the role of this protein in mast cells, the cell type in which we originally discovered it. We investigated the surface expression of εBP in various mast cell lines and addressed the questions of how the protein is bound to the cell surface and whether the surface expression can be modulated. Finally, in a preliminary effort to show the function of εBP, we explored the role of this protein in cell-cell interactions.

MATERIALS AND METHODS

Cells and reagents. Unless otherwise specified, all common reagents were used as obtained from Sigma Chemical Co., St. Louis, MO. εBP (15) and mouse monoclonal anti-DNP IgE (HI-DNP-ε-26.82) (16) were prepared as previously described.

The following cell lines were used for this study: DA-1 (17), is an IL-3-dependent mouse cell line (from Dr. J. Ohara, University of Colorado, Department of Medicine, Denver, CO), and PT-18 is a mouse mast cell line described by Pluznik et al. (18). Mouse bone marrow-derived mast cells were generously provided by the laboratory of Dr. S. Wasserman (University of California, San Diego, Medical School, San Diego, CA). The IL-3-dependent cells were maintained in a medium containing 20% WEHI-3 supernatant (19). HC/3 mouse mast cells (from Dr. D. Katz, Medical Biology Institute, La Jolla, CA) were cultured in DMEM containing 10% FCS. Rat basophilic leukemia cells (RBL-2H3) originally provided by Dr. B. Baird (Cornell University, Ithaca, NY) were maintained in DMEM containing 20% FCS. Mouse macrophage lines P388D1 (ATCC TIB 63), RAW 264.7 (ATCC TIB 71), and WEHI-3 (ATCC TIB 68) were from American Type Culture Collection (ATCC, Rockville, MD) and were maintained in DMEM with 10% FCS.

TG-M0 were obtained by peritoneal lavage of BALB/c mice (Scripps Clinic and Research Foundation mouse colony, La Jolla, CA) as previously described (20).

Preparation of polyclonal rabbit anti-εBP antibodies. New Zealand albino rabbits were immunized as follows: for primary immunization, 100 μg εBP in CFA was given s.c. on day 0, 50 μg in IFA

Received for publication June 17, 1991.

Accepted for publication November 7, 1991.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by National Institutes of Health Grants AI20958 and AI19747. This is publication no. 6791-MEM of the Department of Molecular and Experimental Medicine.

² Address correspondence and reprint requests to Dr. Luciano G. Frigeri, Department of Molecular and Experimental Medicine, BCR-4, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037-1093.

³ Abbreviations used in this paper: εBP, IgE binding protein; RBL, rat basophilic leukemia cells; CBP 35, carbohydrate binding protein; L-34, β-D-galactoside-binding lectin of *M_r* 34,000; Mac-2, macrophage surface antigen of *M_r* 32,000; TG-M0, thioglycolate-elicited macrophages.

s.c. on days 14 and 28. The first blood samples were obtained 7 days after the last boost. Thereafter, booster immunizations of 50 μ g rBP in IFA were given s.c. Corresponding blood samples were obtained 7 to 10 days after each booster injection. Sera were fractionated by ammonium sulfate precipitation and affinity purified with rBP-Sepharose 4B by a procedure described previously (21). rBP-Sepharose 4B was prepared by conjugating 1.5 mg purified rBP to 1 g of Sepharose 4B (Pharmacia, Uppsala, Sweden). Protein concentration was determined by using the BCA reagent (Pierce Chemical Co., Rockford, IL) according to manufacturer's procedure.

SDS-PAGE and immunoblotting. Cell lysates (5×10^7 cell equivalent/ml) were prepared as previously described (1). Proteins were separated by SDS-PAGE (22), and immunoblotting was done by using a polyvinylidene difluoride transfer membrane (Immobilon P, Millipore, Bedford, MA) and anti-rBP serum (1/1000 dilution) as described previously (23).

Radioiodination. rBP, affinity-purified rabbit anti-rBP antibodies, and goat anti-rabbit antibodies were iodinated by using the chloramine-T method (15). The sp. act. were rBP 1.5×10^{15} cpm/mmol; rabbit anti-rBP antibody, 1.5×10^{15} cpm/mmol; and goat anti-rabbit antibody, 2.0×10^{16} cpm/mmol.

Determination of cell-surface ϵ BP with ^{125} I-anti-rBP. Binding assays were performed by using a phthalate-oil separation method described previously (24). Cells ($1-1.5 \times 10^5$) were mixed with ^{125} I-anti-rBP (0.1 pmol) in 100 μ l of RPMI 1640 containing 1% BSA, 20 mM HEPES buffer, 0.1% sodium azide, and 10 μ g/ml normal rabbit γ -globulin, pH 7.2 (binding buffer). The mixtures were incubated in 1.5-ml Eppendorf test tubes for 4 h at 4°C with occasional shaking. The mixtures were then transferred to precooled 400- μ l polyethylene centrifuge tubes containing a mixture of dibutylphthalate and 15% (v/v) mineral oil, and cell-bound ^{125} I-anti-rBP antibodies were separated from free ^{125} I-anti-rBP by centrifuging the tubes for 30 s in a Beckman Microfuge E (Beckman Instruments Inc., Fullerton, CA). Nonspecific binding was measured in the presence of 100 μ g/ml unlabeled anti-rBP corresponding to a 500-fold molar excess of cold ligand. Each data point was run in duplicate and the experiment was repeated at least twice. Data were analyzed by using an equation for competitive inhibition between two ligands for one type of site (25) and by Scatchard analysis (26).

Elution of surface ϵ BP from P388D1 cells with lactose. Cells (4.0×10^6) were washed twice with binding buffer and divided into two fractions of 2 ml each in the same binding buffer. To one fraction, lactose was added to a final concentration of 25 mM. Tubes were incubated for 10 min at room temperature and then centrifuged 5 min at 1200 rpm. Supernatants were transferred to dialysis bags (8000 M, cut-off, Spectrapore, Los Angeles, CA) containing 100 μ l of lactosyl-Sepharose 4B beads prepared as described previously (27) and dialyzed overnight against 1 liter of 10 mM Tris-HCl, pH 7.5, at 4°C. Beads were recovered by washing the dialysis bag twice with 10 mM Tris-HCl, pH 7.5. Beads were then washed twice with PBS containing 1% Triton X-100, pH 7.2, and twice with 1.5 ml of 62.5 mM Tris-HCl, pH 6.8. Bound ϵ BP was eluted by boiling the beads for 3 min in SDS-PAGE loading buffer (2% SDS, 62.5 mM Tris-HCl, 10% glycerol, 0.01% bromophenol blue, pH 6.8).

Determination of ϵ BP binding sites on cell surface. Two different approaches were used to determine the number of ϵ BP binding sites on the cell surface. In the first, the competitive displacement of ^{125} I-rBP by unlabeled ligand was determined. Cells (2.0×10^5) were incubated with ^{125} I-rBP (1 pmol) in 100 μ l binding buffer in the presence of 0.75 nM to 1.5 μ M of unlabeled rBP under the same conditions described for the determination of cell surface ϵ BP with ^{125} I-anti-rBP. Nonspecific binding was measured in the presence of 25 mM lactose. Assays were run in duplicate. In the second approach, the number of ϵ BP binding sites was determined as in the first approach by measuring the total ^{125} I-rBP specifically bound under saturating conditions in the absence of unlabeled ϵ BP. Four replicates were used for each data point.

Activation of RBL-2H3 cells and determination of cell surface ϵ BP. Cells were harvested, resuspended in DMEM 20% FBS at 3×10^6 cells/ml, and divided into two fractions. The following additions were made: fraction A: 10 μ l/ml [^{3} H]5 hydroxytryptamine binoxalate (1 mCi/ml, 26.7 Ci/mmol) and mouse anti-DNP IgE at 0.1 μ g/ml final concentration; fraction B: mouse anti-DNP IgE (0.1 μ g/ml) without radioactive serotonin precursor. Both fractions were incubated with gentle agitation for 1 h at 37°C, centrifuged briefly, and washed three times with DMEM-20% FBS. Cells from fraction A were used to determine [^{3} H]serotonin release as previously described (28). Cells from fraction B were centrifuged one more time in Tyrode's buffer (137 mM NaCl, 5.6 mM glucose, 2.7 mM KCl, 0.4 mM NaH₂PO₄, 10 mM HEPES, 0.1% gelatin, pH 7.5), and then aliquots were dispensed into several test tubes containing appropriate amounts of DNP₃₀-BSA in Tyrode's buffer supplemented with CaCl₂ (0.9 mM) and MgCl₂ (0.1 mM). The tubes were incubated at 37°C for 45 min and centri-

fuged briefly at 4°C to terminate the activation. Cells were washed once in binding buffer, counted in a Weiss chamber, and resuspended in the same binding buffer at appropriate concentrations. Surface ϵ BP was determined by using the ^{125} I-anti-rBP antibody as described earlier. Statistical significance was estimated by using one-way analysis of variance by ranks (Kruskal-Wallis) for nonparametric analysis.

Rosette assay. Trypsin-treated and glutaraldehyde-fixed rabbit E (from NZW rabbits, Scripps Clinic and Research Foundation rabbit colony) were prepared as described by Nowak et al. (29). Cultured cells were resuspended in DMEM 10% FCS at 2×10^6 cells/ml, and 50- μ l aliquots were mixed with equal volumes (50 μ l) of trypsinized E (1% suspension in DMEM 10% FCS) in 96-well round-bottom microtiter plates (Microtiter III, Dynatech, Chantilly, VA) coated with 3% BSA. The cell mixtures were centrifuged at $90 \times g$ for 3 min and kept on ice for 2 h. Pellets were gently suspended with a Pasteur pipette, and aliquots were diluted in Trypan blue (0.1% in PBS) before examination under a light microscope. Treatment of cells with lactose before the rosette assay consisted of a 10- to 15-min incubation of the cells in DMEM 10% FBS containing 25 mM lactose followed by washing (twice) in the same buffer. For the rosette assays of these cells, lactose was also present during the 2-h incubation at 4°C with the E. An aliquot of cells treated with lactose was reexposed to rBP by incubating the cells for 10 to 15 min with 10 μ g/ml of the recombinant protein and then washing them three times in DMEM 10% FCS to remove excess rBP. Four replicates were used for each data point. Statistical significance was determined by using one-way analysis of variance by ranks (Kruskal-Wallis) and multiple range test for nonparametric analysis.

RESULTS

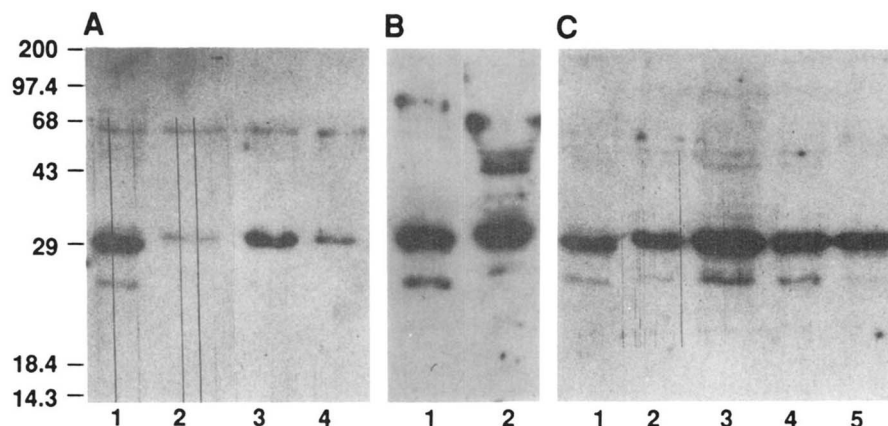
Different mast cell lines express variable amounts of ϵ BP. As mentioned earlier, we now know that ϵ BP is identical to Mac-2. Early work indicated that Mac-2 expression increases when macrophages undergo maturation (30). Further, Mac-2 is found in relatively low quantities in resting macrophages, but its expression is dramatically increased in macrophages elicited by inflammatory stimuli, such as thioglycollate (13). Because ϵ BP is present abundantly in RBL cells (a mucosal mast cell type), in which the protein was initially identified and from which the cDNA was cloned, we wished to compare the expression of ϵ BP in different mast cell lines.

First, the total amount of ϵ BP in the various cell lines was compared by using immunoblotting and quantitated by densitometry scanning. In these experiments, cell lysates from equal numbers of various mast cell lines were subjected to electrophoresis, the separated proteins were transferred to membranes, and ϵ BP was detected by using antibodies to the recombinant ϵ BP. As shown in Figure 1, A and B, expression of ϵ BP varied among the mast cell lines. The PT-18 line (Fig. 1B, lane 2) expressed the highest amount. Next were RBL (Fig. 1A, lane 1 and B, lane 1), HC/3 (A, lane 3), and bone marrow-derived mast cells (A, lane 4). DA-1, a IL-3 dependent lymphoblastoma line, also expressed a relatively low level of ϵ BP (Fig. 1A, lane 2).

Several macrophage lines were included for comparison. ϵ BP was expressed in all the macrophages lines analyzed, and levels of ϵ BP were comparable among the lines. With the exception of WEHI-3 (Fig. 1C, lane 3), which expressed the highest level of ϵ BP of all the cells studied, the amounts of ϵ BP in other macrophage cell lines, including P388D1 (C, lane 2), RAW 264.7 (C, lane 4), and TG-M0 (C, lane 5) were similar to the amount found in RBL cells (C, lane 1).

ϵ BP is expressed on surface of mast cells and macrophages. Inasmuch as the discovery of ϵ BP, we have asked whether the protein is expressed on the cell surface, as the answer has an obvious impact on the func-

Figure 1. Immunoblot analysis of total ϵ BP content of various mast cells and macrophage lines. Aliquots of lysates of various cell lines (5×10^5 cell equivalent) were electrophoresed on 12.5% SDS-polyacrylamide gels, and the proteins were electroblotted to Immobilon membranes. ϵ BP was probed with rabbit anti- ϵ BP antibodies followed by ^{125} I-labeled goat anti-rabbit antibodies. The immunoblot was quantitated using a laser scanner (LKB Ultrosan XL) and the intensity of the band relative to RBL is reported below in parentheses. **A**, Lane 1, RBL cells (1.0); lane 2, DA-1 (0.72) cells; lane 3, HC/3 (0.084) cells; lane 4, bone marrow-derived mast cells (0.45). **B**, Lane 1, RBL cells (1.0); lane 2, PT-18 cells (1.95). **C**, Lane 1, RBL cells (1.0); lane 2, P388D1 (1.28); lane 3, WEHI-3 (2.95); lane 4, RAW 264.7 (2.16); lane 5, thioglycolate-elicited mouse macrophages (1.87). The cell lysate used for each lane corresponded to 5×10^5 cells. The immunoblot analysis of the PT-18 cells was repeated three times and the intensity of the band relative to RBL was 6.74, 1.95, 1.20. Anti-r ϵ BP serum was used at 1/1000 dilution. ^{125} I-labeled goat anti-rabbit antibody was used at 5×10^5 cpm/ml. Positions of m.w. standards ($\times 10^{-3}$) are shown at left. Exposure time of the autoradiographs was 20 h at -75°C .



tional assessment of ϵ BP. Previous studies with antibodies to a synthetic peptide corresponding to an internal sequence of ϵ BP showed that the protein was present on the surface of RBL cells (23). However, in that study, it was concluded that ϵ BP is primarily a cytoplasmic protein. Studies with a mAb also established that Mac-2 is expressed on the cell surface. TG-M0 contain as much as 1.7×10^5 molecules of Mac-2 on the cell surface (13). Further, various macrophage cell lines express variable amounts of Mac-2 on their cell surfaces, and the amount appears to correlate with maturation of macrophages (30). The availability of polyclonal antibodies against r ϵ BP made it possible to quantify the amount of ϵ BP on the cell surfaces of various cell lines.

First, we assessed the usefulness of using affinity-purified anti- ϵ BP antibodies to quantify the amount of cell-surface ϵ BP on the P388D1 macrophage line, which has been shown to express a relatively high amount of Mac-2 on its surface (30). As shown in Figure 2A, ^{125} I-anti-r ϵ BP bound specifically to P388D1 cells, and the binding was inhibited by increasing concentrations of unlabeled antibodies. Scatchard analysis of the data (Fig. 2B) showed a nonlinearity, most likely reflecting the presence of monovalent (low affinity) and bivalent (high affinity) bound states of the antibodies, as previously described for this type of analysis (24). The number of ϵ BP molecules/cell was estimated by extrapolation of the low affinity binding curve of the Scatchard plot. From this analysis, the number of ϵ BP molecules on the surface of a P388D1 cell was determined to be 3.54×10^4 , with K_d of $2.5 \times 10^8 \text{ M}^{-1}$.

The amounts of ϵ BP on the cell surfaces of mast cell lines and other macrophage lines were similarly analyzed; the results are shown in Figure 3. It is evident that the amount of ϵ BP on the cell surface varied widely. The corresponding values, in descending order, were: TG-M0 (1.04×10^5 sites/cell) > P388D1 (3.54×10^4) > WEHI-3 (1.34×10^4) = RBL (1.09×10^4) > PT-18 and HC/3 ($<1.0 \times 10^3$). The value for TG-M0 is in close agreement with that previously reported for Mac-2 (1.7×10^5 sites/cell), quantified by using a mAb (13). The relative values for TG-M0, P388D1, and WEHI-3 also agree with those previously reported for the level of Mac-2 on these cells as

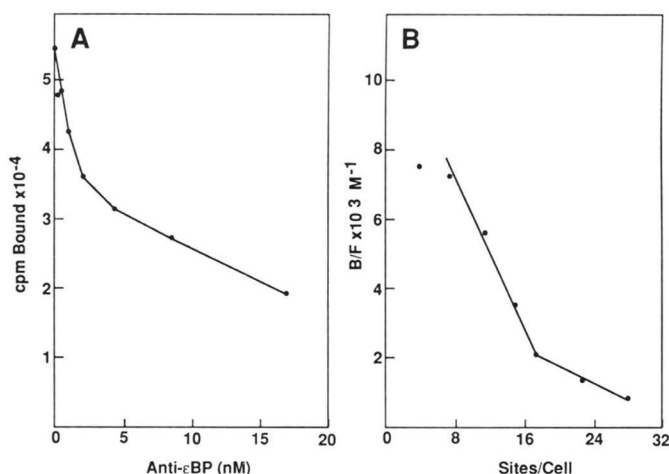


Figure 2. Scatchard analysis of the number of cell surface ϵ BP molecules on P388D1 macrophages. **A**, Inhibition of ^{125} I-anti- ϵ BP binding to P388D1 cells by unlabeled anti- ϵ BP. Cells (1.5×10^5) were incubated with ^{125} I-anti-r ϵ BP (1.11×10^{-9}) and various concentrations of unlabeled anti-r ϵ BP for 4 h at 4°C , and bound and free radiolabeled antibodies were determined as described in *Materials and Methods*. Nonspecific binding was measured in the presence of 500-fold excess of unlabeled anti- ϵ BP and found to be less than 10% of the total ^{125} I-labeled anti- ϵ BP antibody bound. **B**, Scatchard representation of the binding data presented in **A**. The number of ϵ BP molecules was derived by extrapolation of the lower curve, which represent the low affinity binding.

determined by flow cytometric analysis (30).

These results confirmed that ϵ BP is expressed on the surface of RBL cells. However, a more interesting finding was that the amount of ϵ BP on the surface of various mast cell lines did not correlate with the total cellular amount of ϵ BP. Thus, although PT-18 cells have a higher total amount of ϵ BP than RBL cells do (Fig. 1B), the former cells have hardly any detectable surface ϵ BP. A similar conclusion was made for the macrophage cell lines (compare Fig. 1 and Fig. 3).

Cell surface ϵ BP can be detached from cells by lactose treatment. Once we had established that ϵ BP is expressed on the surface of mast cells (and macrophages), the next question was, how is the protein expressed? The question is not trivial, because the protein sequence of ϵ BP deduced from the cDNA sequence does not contain any obvious transmembrane segment. Inasmuch as ϵ BP is a lectin, it

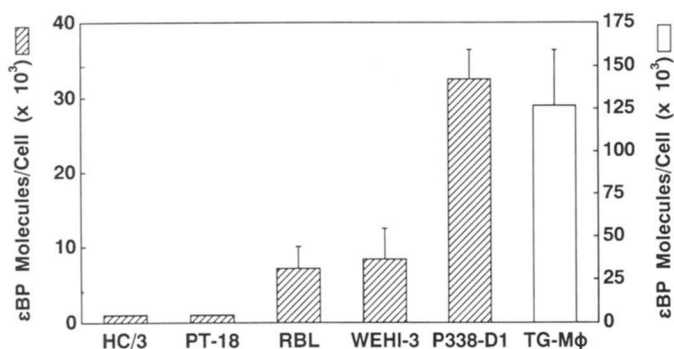


Figure 3. Amount of cell-surface ϵ BP in various cell lines. The number of ϵ BP molecules per cell for various cell lines was determined by using ^{125}I -anti- ϵ BP antibody as described in Figure 2. Scatchard plots were obtained for each cell line and from which the number of ϵ BP molecules per cell was derived as described in Figure 2. The scales on the left y-axis are used for the solid bars, and those on the right y-axis are used for the open bar. Values are mean $\pm$ SD. The number of surface ϵ BP molecules on RBL cells is the average of four separate experiments. For the remaining cell types, results from two experiments were averaged.

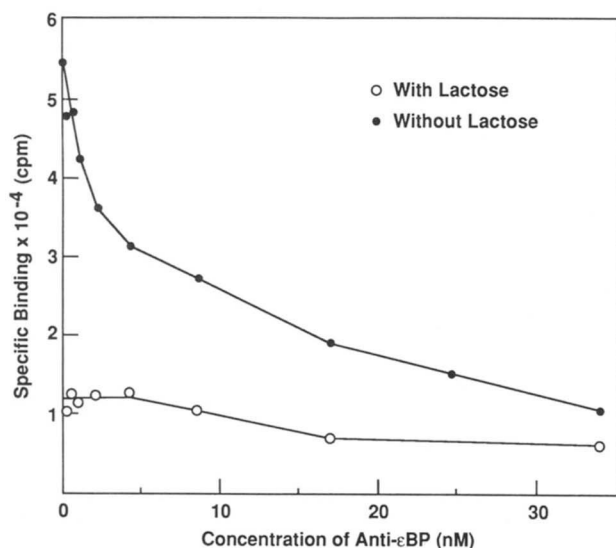


Figure 4. Binding of anti- ϵ BP to P388D1 in presence or absence of lactose. Cultured P388D1 cells were incubated in a medium in the presence or absence of 25 mM lactose for 15–20 min at 24°C, then the analysis of cell surface ϵ BP was made with ^{125}I -labeled antibody in the presence or absence of 25 mM lactose. The binding conditions and analysis were as described in Figure 2 and Materials and Methods.

might be attached to other glycoproteins or glycolipids on the cell surface. If so, it should be possible to detach ϵ BP by treating cells with lactose. P388D1 cells were incubated with 25 mM lactose for 10 min at 24°C, and their surface level of ϵ BP was determined as before by using ^{125}I -labeled anti- ϵ BP antibodies in presence of lactose. As shown in Figure 4, binding of ^{125}I -anti- ϵ BP was reduced nearly to background levels in the presence of lactose. Similar results were obtained with RBL, WEHI-3, RAW 267.3, and TG-M0 cells and in each case binding of ^{125}I -labeled antibodies was almost abolished. When P388D1 cells were pretreated with lactose (30 min at 24°C), washed, and exposed to ^{125}I -labeled antibody in the absence of lactose the number of cell surface ϵ BP was found to be 36% of untreated control.

To show that these results reflected the detachment of ϵ BP from the cell surface, we analyzed the supernatant fluid of P388D1 cells treated with lactose for the presence of ϵ BP. We used affinity purification of the protein with lactosyl-Sepharose 4B followed by immunoblotting with

specific anti- ϵ BP antibodies. As shown in Figure 5, a protein of M_r 32,000 was detected in the supernatant fluids of P388D1 cells treated with lactose (lane 3); a similar protein was not found in the supernatant fluid from untreated cells (lane 2). Figure 5, lane 1 shows ϵ BP used as a reference for the immunoblot. The slight difference in apparent m.w. between the bands in Figure 5 lanes 1 and 3 most likely reflects the fact that P388D1 is a cell line derived from a mouse, and mouse ϵ BP has a slightly higher m.w. than rat ϵ BP (5). The removal of cell surface ϵ BP on RBL cells by lactose was also confirmed after the same approach (data not shown). The results strongly suggested that lactose causes dissociation of ϵ BP from the cell surface. On the basis of these analyses, we concluded that ϵ BP is attached to cell-surface glycoconjugates by virtue of its lectin property and that this attachment is inhibitable by lactose.

Mast cells and macrophages have comparable numbers of binding sites for ϵ BP and variation in surface levels of ϵ BP is not dictated by number of ϵ BP-binding sites. We wished to establish if the variability in the amount of ϵ BP expressed on the cell surface in the different cell lines reflected a variability in the number of "sites" that ϵ BP binds to. For this purpose, ϵ BP was iodinated, and the amount of ϵ BP bound to the cells under saturating conditions (B_0) was quantified by competitive displacement of labeled ligand as described in Materials and Methods. The analysis indicated that for RBL cells the number of ϵ BP binding sites was 7.45×10^4 sites/cell, with a K_d of $1.97 \times 10^8 \text{ mol}^{-1}$. The data also showed that under the experimental conditions chosen a similar

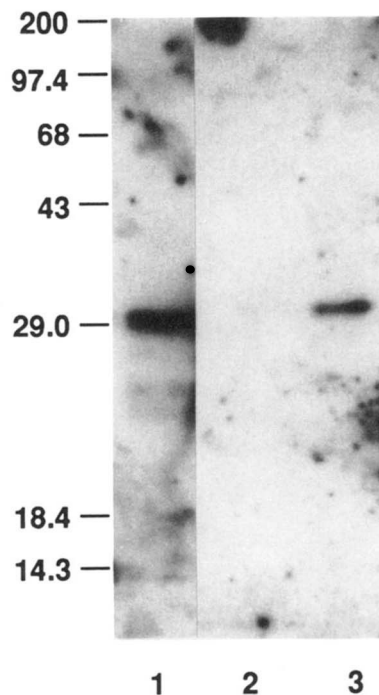


Figure 5. Immunoblot analysis of ϵ BP eluted from P388D1 cells with lactose. Supernatant fluid from P388D1 cells either untreated or treated with lactose was mixed with lactosyl-Sepharose, and the bound proteins were eluted and analyzed by immunoblotting. Lane 1, ϵ BP. Lane 2, protein affinity purified from the supernatant fluid of cells incubated in binding buffer only. Lane 3, protein purified from the supernatant fluid of cells incubated in the presence of 25 mM lactose in the binding buffer. Blots were developed with anti-rat ϵ BP serum and ^{125}I -goat anti-rabbit antibody as described in Figure 1. Numbers on the left denote positions of m.w. markers ($\times 10^{-3}$). The time of exposure of the autoradiograph was 20 h at -75°C .

value for the number of ϵ BP binding sites was obtained when the total amount of ^{125}I - ϵ BP specifically bound in the absence of cold ligand was determined ($B_0 = 8.96 \times 10^4$ sites/cell). Therefore, we determined the number of ϵ BP binding sites in two other cell lines, PT-18 and P388D1, by simply measuring the total amount of ^{125}I - ϵ BP specifically bound under saturating conditions. The results indicated that the three cell lines have a similar number of ϵ BP binding sites (Fig. 6): P388D1, 10.2×10^4 sites/cell, RBL, 8.96×10^4 sites/cell, and PT-18, 4.9×10^4 sites/cell. Similar numbers were obtained with cells treated with lactose to remove endogenous ϵ BP, indicating that ^{125}I - ϵ BP, used in excess of the ϵ BP initially present on the cell surface, must have displaced the endogenous ϵ BP under these experimental conditions. When the number of binding sites per cell was compared with the amount of ϵ BP present on the cell surface in each of the cell types (Fig. 3 vs Fig. 6), it became evident that the number of available binding sites for ϵ BP in these cells was in excess of the amount of ϵ BP on the surface and that for cell lines such as PT-18 the lack of detectable surface ϵ BP was not due to a limited number of binding sites for ϵ BP.

Surface level of ϵ BP on RBL cells increases when cells are activated. We had noted a variation in the surface levels of ϵ BP in various mast cell lines, and we wished to determine the basis for it. In the case of macrophages, the expression of Mac-2 (ϵ BP) was significantly elevated under the influence of certain inflammatory stimuli (13). We reasoned that inasmuch as the number of ϵ BP-binding sites on RBL cells exceeds the number of molecules of ϵ BP on the cell surface, these cells could have more ϵ BP on the surface under certain conditions, such as IgE-dependent activation mediated by cross-linking of the high-affinity IgE receptor (Fc ϵ R1). Thus, RBL cells were sensitized with mouse monoclonal anti-DNP IgE and then activated with DNP-BSA. The degree of degranulation was determined by measuring the release of serotonin, and the amount of ϵ BP on the cell surfaces of the activated cells was assessed with ^{125}I -anti- ϵ BP antibody. As shown in Figure 7, when the cells were activated with increasing

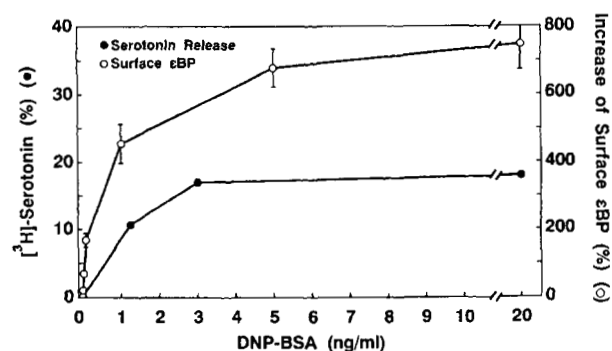


Figure 7. Activation of RBL-2H3 and expression of ϵ BP on the cell surface. Serotonin release: RBL-2H3 cells were sensitized in the presence of $0.1 \mu\text{g/ml}$ anti-DNP IgE and $10 \mu\text{g/ml}$ of [^3H]serotonin precursor and subsequently treated with various concentrations of DNP₃₀-BSA. Percentage of serotonin release was determined as described in *Materials and Methods*. Data are mean $\pm$ SD of two replicates. Expression of cell surface ϵ BP: RBL-2H3 cells were sensitized as above in the absence of radiolabeled serotonin precursor and activated with various doses of DNP₃₀-BSA. Surface expression of ϵ BP was determined by analysis of binding with ^{125}I -anti- ϵ BP antibody as described in *Materials and Methods*. Data are mean $\pm$ SD of four determinations. Statistical significance was determined by using one-way analysis of variance by ranks (Kruskal-Wallis), $p < 0.025$. The experiment was repeated five times.

amounts of DNP-BSA, a dose-dependent release of serotonin was observed concomitantly with a statistically significant ($p < 0.025$) increase of cell surface ϵ BP. The probability value of $p < 0.025$ was calculated using one-way analysis of variance by ranks (Kruskal-Wallis) where the number of cell surface ϵ BP molecules on mast cells sensitized with IgE and subsequently triggered with DNP-BSA was compared with those on cells sensitized but not triggered, taking into consideration all doses of DNP-BSA. We therefore concluded that under the influence of certain physiologic stimuli, the surface expression of ϵ BP in mast cells can be modulated.

Cell-surface ϵ BP is functional. Having established that ϵ BP is expressed on the cell surface of mast cells (and macrophages) and having noted that the level of ϵ BP can be increased under certain conditions, we wished to know the function of this protein on the cell surface. Because it binds IgE, it is possible that ϵ BP may participate in IgE sensitization of mast cells. In broader terms, because ϵ BP is a lectin, it might have a role in cell-cell or cell-matrix interactions through recognition of glycoconjugates. As we have concluded that ϵ BP is expressed on the cell surface by virtue of its lectin activity, it is questionable that ϵ BP on the cell surface can simultaneously bind other glycoconjugates. However, we previously showed that ϵ BP could cause hemagglutination of trypsinized E (15), suggesting that, as with many other lectins, ϵ BP may be bivalent or multivalent with regard to this carbohydrate-binding capacity. As a first approach to show that cell-surface ϵ BP was functional, we tested the capacity of cells with ϵ BP on their surfaces to form rosettes with trypsinized erythrocytes.

As shown in Table I, with P388D1 and RBL cells, nearly 40% of the cells formed rosettes. In significant contrast, PT-18 cells, which lack detectable surface ϵ BP, formed much fewer rosettes (10%) when tested under the same conditions. To show the role of ϵ BP in the formation of rosettes, we tested the ability of anti- ϵ BP antibodies to inhibit the process. The percentage of rosette-forming cells was not reduced significantly. It was reasoned that anti- ϵ BP antibodies might not block the active site of ϵ BP.

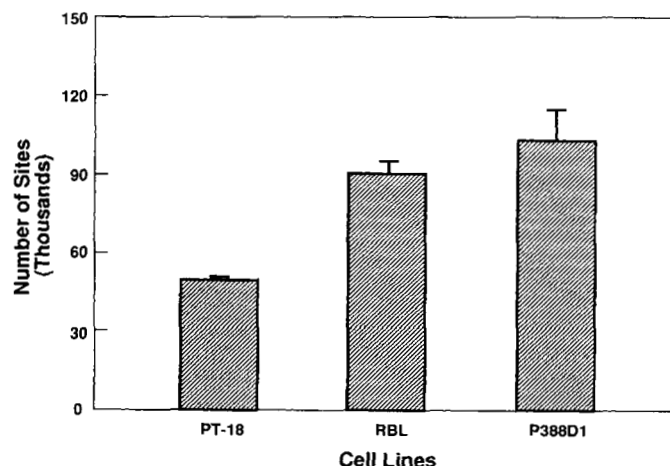


Figure 6. Number of surface ϵ BP-binding sites on various cell lines. Binding of ^{125}I - ϵ BP to various cells was performed by using the conditions described in *Materials and Methods*. The concentration of ^{125}I - ϵ BP was $1.67 \times 10^{-6}\text{M}$ (sp. act., 1.5×10^{15} cpm/mmol). Nonspecific binding was measured in the presence of 25 mM lactose. The ϵ BP-binding sites were calculated from the total amount of ^{125}I - ϵ BP specifically bound. Quadruplicate determinations were performed for each cell type. Data represent the mean $\pm$ SD. The experiment was repeated twice.

TABLE I

Expression of functional surface ϵ BP on P388-D1 and RBL cell lines as assessed by rosetting with rabbit E

Cells	Treatment	Percent Rosettes ^a
P388-D1	None	39.6 $\pm$ 8.1
P388-D1	Lactose ^b	18.1 $\pm$ 2.0 ^c
P388-D1	Lactose + ϵ BP ^d	35.8 $\pm$ 6.2
RBL	None	36.2 $\pm$ 4.8
RBL	Lactose ^b	21.5 $\pm$ 2.1 ^c
RBL	Lactose + ϵ BP ^d	35.1 $\pm$ 6.8
PT-18	None	11.4 $\pm$ 2.4

^a Treated and untreated cells were mixed with rabbit E in the presence (for lactose-treated cells) or absence (for untreated cells and cells first treated with lactose and subsequently exposed to ϵ BP) of 25 mM lactose, and rosette-forming cells were counted. A total of 175 to 220 cells were counted for the P388D1 and PT-18 groups, and 490-743 cells were counted for the RBL groups. Each group of data represents the mean $\pm$ SD of four determinations.

^b P388D1 or RBL cells were treated with medium containing 25 mM lactose to elute cell-surface ϵ BP.

^c $p < 0.005$ compared with untreated cells. The experiment was repeated twice.

^d Lactose-treated cells were incubated in medium containing 10 μ g/ml of rat ϵ BP.

We then used cells treated with lactose before the assay, which, as shown earlier, removes a substantial proportion of the cell-surface ϵ BP. As shown in Table I, cells treated with lactose formed fewer rosettes than untreated cells did. Further support for the role of ϵ BP in rosetting was provided by the results of experiments in which cells were treated with lactose and subsequently exposed to ϵ BP. According to the data presented earlier in this report, these cells should have regained their cell surface ϵ BP. In fact, the rosette-forming capacity of these cells was restored (Table I).

We concluded that rosette formation by the cells studied was mediated, at least in part, by surface ϵ BP, most likely via the protein's carbohydrate-binding activity. We do not know why removal of surface ϵ BP with lactose did not completely abolish rosette formation (approximately 20% residual). Probably, this was partially due to residual ϵ BP bound to the cell surface, as the amount of ¹²⁵I-labeled anti- ϵ BP antibodies that bound to cells treated with lactose was about 12% of the amount that bound to untreated cells (Fig. 4). In addition, other cell-surface molecules may participate in rosette formation.

DISCUSSION

On the basis of the results presented in this report, we made the following conclusions. 1) ϵ BP is expressed in various amounts by different mast cell lines. 2) ϵ BP is expressed on the cell surface of mast cells. However, the level of surface ϵ BP is not correlated with the amount of ϵ BP in cells. 3) ϵ BP found on the surface of mast cells (and macrophages) appears to be bound to glycoconjugates in a fashion that is reversible by lactose. 4) ϵ BP-binding sites, demonstrable with radiolabeled ϵ BP, are present in comparable levels in various mast cell and macrophage lines. 5) Surface expression of ϵ BP on RBL cells can be modulated. The level of ϵ BP is increased when the cells are activated through the high-affinity IgE receptor by IgE immune complexes. 6) Cell surface ϵ BP on mast cells and macrophages is functional as shown by its role in the formation of rosettes of these cells with E.

The finding that the amount of ϵ BP varies among sev-

eral mast cell lines is interesting. Expression of ϵ BP may depend on the mast cell type (e.g., mucosal cell type vs. serosal cell type) and may be linked to functional distinction of different cell types. Inasmuch as ϵ BP is a lectin, it might be a differentiation marker for mast cells. Determination of a definitive association between expression of ϵ BP and subtypes of mast cells will require more extensive analysis of other cell lines and mast cells from different sources, including characterization of the cell lines with regard to their glycosaminoglycan content, number of granules, and types of proteases.

Previously, we showed that ϵ BP is present on the cell surface of RBL cells (23). Our data confirm that observation and provide further information on the number of cell surface ϵ BP molecules on various mast cell lines (and macrophages). It was important to establish the surface localization of ϵ BP, as this protein does not have a signal sequence or a transmembrane segment. Because ϵ BP does not have a classical signal sequence, it is not clear how the protein gets to the extracellular space. Possibly, it is transported through a novel pathway for proteins that lack a signal sequence, such as IL-1 (31).

The lack of a characteristic transmembrane segment makes ϵ BP a rather unusual cell-surface protein, and thus it is not immediately obvious how the protein is displayed on the cell surface. Our data suggest that it is attached to glycolipids or glycoproteins, most likely involving the galactose residues, as the binding is reversed by lactose. Although these putative attachment sites have yet to be characterized, it is remarkable that a saturable number of ϵ BP-binding sites are present on a number of cell types (Fig. 6) and that the binding of exogenous ϵ BP to these cells can be readily demonstrated. These findings have at least two implications: 1) The expression of cell surface ϵ BP depends on the glycoconjugates, and 3) the surface of the mast cell (or macrophage) has two sources of ϵ BP: an endogenous one transported from an intracellular pool of the cell in which the ϵ BP resides and an exogenous one acquired from the ϵ BP secreted by other cells. Previously, it was shown that TG-M0 released Mac-2 into the medium (8) and using immunoblotting and an anti- ϵ BP antibody, we have detected ϵ BP in the peritoneal fluids in mice treated with thioglycolate (L. G. Frigeri, unpublished observations). Both of these findings support the existence of an exogenous pool of ϵ BP. Thus, our current model for extracellular expression of ϵ BP includes extracellular transport by an undefined mechanism. Subsequently, ϵ BP can remain attached to the original cells, remain in the extracellular fluid, or be acquired by neighboring cells.

Our finding that mast cell lines, such as PT-18, that contain substantial amounts of ϵ BP do not necessarily express ϵ BP on the cell surface suggests that surface localization of ϵ BP is controlled. Interestingly, flow cytometric analysis showed that the surface expression of Mac-2 was linked to maturation of macrophages (30). We found that macrophage cell lines at different degrees of maturation contained comparable amounts of total ϵ BP, again suggesting some sort of controlling mechanism for surface expression that may be related to cellular differentiation.

The finding that RBL cells activated by IgE immune complexes displayed increased amounts of ϵ BP on their surfaces in a manner that depended on the dose of the

stimuli and paralleled the extent of degranulation provides more evidence that expression of ϵ BP on the cell surface is regulated. We do not know the mechanism for the observed up-regulation of ϵ BP expression, but the correlation of increased levels of ϵ BP on the cell surface with the release of the granule contents suggests that these two processes may share a common pathway. In fact, ϵ BP may be associated with the granules.

Finally, the results of studies on the formation of rosettes are evidence that ϵ BP is functional. The implication that ϵ BP has a role in such a process is based on the following. 1) The rosette-forming capacity of various cell lines is correlated with the level of surface ϵ BP on these cells, in particular, the mast cell lines PT-18 and RBL. PT-18 cells have much lower levels of surface ϵ BP than RBL cells do, and PT-18 cells also have a much lower capacity to form rosettes. 2) The percentage of cells forming rosettes is greatly reduced when the cells are treated with lactose, a process known to remove ϵ BP. 3) Rosette-forming capacity can be restored when the cells treated with lactose are subsequently exposed to ϵ BP. This last approach is unique as, unlike most other typical cell surface receptors, cell surface levels of ϵ BP can be easily manipulated artificially by treatment with lactose and ϵ BP. Although we do not have formal proof that exogenously acquired ϵ BP resembles endogenous ϵ BP, as long as ϵ BP can be acquired, as shown by the binding of ϵ BP to various cells, the relevance of the rosette-forming capacity of cells with exogenously added ϵ BP should be quite evident.

As mentioned in the introduction, the protein designated herein as ϵ BP and studied by another group has been implicated in tumor metastasis; the level of expression of this lectin can be correlated with metastatic potential of melanomas (7). Most recently, studies with cells transfected with cloned cDNA provided evidence for the role of this lectin in tumor metastasis (32). The identity of ϵ BP to nonintegrin laminin-binding protein (14) suggests a possible role of ϵ BP in cell adhesion to basement membranes. It has been reported that activation of the mast cell line PT-18 and of bone marrow-derived mast cells resulted in increased adhesion to laminin (33). Although other proteins may be involved, ϵ BP may play a role in such a process. With the information in the literature and our data, a picture is emerging that suggests that ϵ BP (Mac-2) is a new class of cell-adhesion protein.

Acknowledgments. Thanks are due to Dr. Christine A. Gritzmacher for critical reading and to Velda Comstock for her help in preparing the manuscript.

REFERENCES

- Liu, F. -T., and N. Orida. 1984. Synthesis of surface immunoglobulin E receptor in *Xenopus* oocytes by translation of mRNA from rat basophilic leukemia cells. *J. Biol. Chem.* 259:10649.
- Liu, F. -T., K. Albrandt, E. Mendel, A. Kulczycki, Jr., and N. K. Orida. 1985. Identification of an IgE-binding protein by molecular cloning. *Proc. Natl. Acad. Sci. USA* 82:4100.
- Albrandt, K., N. K. Orida, and F. -T. Liu. 1987. An IgE-binding protein with a distinctive repetitive sequence and homology with an IgG receptor. *Proc. Natl. Acad. Sci. USA* 84:6859.
- Liu, F. -T. 1990. Molecular biology of IgE-binding protein, IgE-binding factors and IgE receptors. *CRC Crit. Rev. Immunol.* 10:289.
- Laing, J. G., M. W. Robertson, C. A. Gritzmacher, J. L. Wang, and F. -T. Liu. 1989. Biochemical and immunological comparisons of carbohydrate-binding protein 35 and an IgE-binding protein. *J. Biol. Chem.* 264:1907.
- Leffler, H., F. R. Masiarz, and S. H. Barondes. 1989. Soluble lactose-binding vertebrate lectins: a growing family. *Biochemistry* 28:9222.
- Raz, A., G. Pazerini, and P. Carmi. 1989. Identification of the metastasis-associated, galactoside-binding lectin as a chimeric gene product with homology to an IgE-binding protein. *Cancer Res.* 49:3489.
- Cherayil, B. J., S. J. Weiner, and S. Pillai. 1989. The Mac-2 antigen is a galactose-specific lectin that binds IgE. *J. Exp. Med.* 170:1959.
- Drickamer, K. 1988. Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* 263:9557.
- Moutsatsos, I. K., M. Wade, M. Schindler, and J. L. Wang. 1987. Endogenous lectins from cultured cells: Nuclear localization of carbohydrate-binding protein 35 in proliferating 3T3 fibroblasts. *Proc. Natl. Acad. Sci. USA* 84:6452.
- Laing, J. G., and J. L. Wang. 1988. Identification of carbohydrate binding protein 35 in heterogeneous nuclear ribonucleoprotein complex. *Biochemistry* 27:5329.
- Raz, A., and R. Lotan. 1987. Endogenous galactoside-binding lectins: a new class of functional tumor cell surface molecules related to metastasis. *Cancer Metast. Rev.* 6:433.
- Ho, M. -K., and T. A. Springer. 1982. Mac-2, a novel 32,000 Mr mouse macrophage subpopulation-specific antigen defined by monoclonal antibodies. *J. Immunol.* 128:1221.
- Woo, H. J., L. M. Shaw, J. M. Messier, and A. M. Mercurio. 1990. The major non-integrin laminin binding protein of macrophages is identical to carbohydrate binding protein 35 (Mac-2). *J. Biol. Chem.* 265:7097.
- Frigeri, L. G., M. W. Robertson, and F. -T. Liu. 1990. Expression of biologically active recombinant rat IgE-binding protein in *Escherichia coli*. *J. Biol. Chem.* 265:20763.
- Liu, F. -T., J. W. Bohn, E. L. Ferry, H. Yamamoto, C. A. Molinaro, L. A. Sherman, N. R. Klinman, and D. H. Katz. 1980. Monoclonal dinitrophenyl-specific murine IgE antibody: preparation, isolation and characterization. *J. Immunol.* 124:2728.
- Pierce, J. H., P. P. diFiore, S. A. Aaronson, M. Potter, J. Pumphrey, A. Scott, and J. H. Ihle. 1985. Neoplastic transformation of mast cells by Abelson-MuLV: abrogation of IL-3 dependence by a nonautocrine mechanism. *Cell* 41:685.
- Pluznik, D. H., N. S. Tare, M. M. Zatz, and A. L. Goldstein. 1982. A mast/basophil cell line dependent on colony stimulating factor. *Exp. Hematol.* 10(Suppl. 12):211.
- Razin, E., J. M. Mencia-Huert, R. L. Stevens, R. A. Lewis, F. -T. Liu, E. T. Corey, and F. K. Austen. 1983. IgE mediated release of leukotriene C₄, chondroitin sulfate E, proteoglycan, β -hexosaminidase, and histamine from cultured bone marrow-derived mouse mast cells. *J. Exp. Med.* 157:189.
- Ralph, P., M. -K. Ho, B. Litcfsky, and T. A. Springer. 1983. Expression and induction in vitro of macrophage differentiation antigen on murine cell lines. *J. Immunol.* 130:108.
- Robertson, M. W., and F. -T. Liu. 1988. IgE structure-function relationships defined by sequence directed antibodies induced by synthetic peptides. *Mol. Immunol.* 25:103.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680.
- Gritzmacher, C. A., M. W. Robertson, and F. -T. Liu. 1988. IgE-binding protein subcellular location and gene expression in many murine tissues and cells. *J. Immunol.* 141:2801.
- Dower, S. K., K. Ozato, and D. M. Segal. 1984. The interaction of monoclonal antibodies with MHC class I antigens on mouse spleen cells. 1. Analysis of the mechanism of binding. *J. Immunol.* 132:751.
- Cuatrecasas, P., and M. D. Hollenberg. 1975. Membrane receptors and hormone actions. *Ad. Prot. Chem.* 30:251.
- Scatchard, G. 1948. The attraction of proteins for small molecules. *Am. N.Y. Acad. Sci.* 51:660.
- Levi, G., and V. I. Teichberg. 1981. Isolation and physicochemical characterization of electrolectin, a β -D-galactoside binding lectin from the electric organ of *Electrophorus electricus*. *J. Biol. Chem.* 256:5735.
- Apgar, J. H. 1990. Antigen-induced cross-linking of the IgE receptor leads to an association with the detergent-insoluble membrane skeleton of rat basophilic leukemia (RBL-2H3) cells. *J. Immunol.* 145:3814.
- Nowak, T. P., D. Kobiler, L. E. Roe, and S. H. Barondes. 1977. Developmentally regulated lectin from embryonic chick pectoral muscle. *J. Biol. Chem.* 252:6026.
- Leenen, P. J. M., A. M. A. C. Jansen, and W. Van Ewijk. 1986. Murine macrophage cell lines can be ordered in a linear differentiation sequence. *Differentiation* 32:157.
- Muesch, A., E. Hartmann, K. Rohde, A. Rubartelli, R. Sitia, and T. A. Rapoport. 1990. A novel pathway for secretory proteins? *Trends Biochem. Sci.* 15:86.
- Raz, A., D. Zhu, V. Hogan, N. Shah, T. Raz, R. Karkash, G. Pazerini, and P. Carmi. 1991. Evidence for the role of 34-kDa galactoside-binding lectin in transformation and metastasis. *Int. J. Cancer* 46:871.
- Thompson, H. L., P. D. Burbelo, B. Segui-Real, Y. Yamada, and D. D. Metcalfe. 1989. Laminin promotes mast cell attachment. *J. Immunol.* 143:2323.