Specific Binding of Antigen to Lymphocytes

Evidence for Lack of Unispecificity in Antigen-Binding Cells

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IT IS NOW A PARADIGM IN IMMUNOBIOLOGY that immunoglobulin-like receptors that are specific for determinants to which the animal had not previously been exposed exist on the surface of lymphoid cells.

This surface position was at first a logical construction required to explain recognition within the allowed bounds of schemes for induction of protein synthesis. More recently, it has become incontrovertible fact, as has been well summarized in a series of articles in reference 1.

In conformity with the classical presentation of the clonal selection theory, the assumption has been widely accepted that antibody-forming cell precursors are restricted to a unique specificity.[2-4] The idea of pluri- or oligospecific precursor cells seems heretical and meets with a great deal of resistance.

There appear to be three main arguments for unispecificity in precursor cells:

1. *Restriction in Antibody-Secreting Cells.* Mäkelä and Cross have recently reviewed the rather formidable evidence that these cells by-and-large produce antibody of single specificity, of a single immunoglobulin class and show allelic exclusion.[2] However, these cells are many generations removed from the precursor cells originally reacting with antigen and there has clearly been ample time for considerable selection and differentiation as in other developing systems.

2. *Specific Deprivation and Tolerance Experiments.* These experiments, which have been used to support unispecificity, simply rule out omnipotence. For example, in the elegant experiments of Ada and Byrt [5] and of Humphrey and Keller [6] on the specific suicide of precur-

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sor cells with very hot antigen, the fact that other immune responses are not affected indicates only that each cells does not retain the ability to respond to a large fraction of the universe of antigenic determinants. The specificity of tolerance merely points to this same limited conclusion. Again, removal of a "single" specificity class of cells on antigen-coated columns without unrelated cells apparently being affected is another instance where the loss of a small minority of cells that might also bind a second test antigen would go unnoticed. (Consider a population of antigen-binding cells each of which bears receptors of five specificities randomly drawn from 1000 possible unique specificities. For an antigen that has ten determinants, one would expect 5% [(10 ÷ 1000) × 5 × 100] of the cells to bind that antigen. Similarly, a second antigen, having ten different determinants, would be bound by 5% of the cells. However, only 0.25% (5% of 5%) of the cells would have receptors for both antigens. Thus, quantitative removal of cells binding one antigen would remove only 5% of the cells binding a second antigen, under this set of circumstances.)

3. *Small Numbers of Specific Antigen-Binding Cells in Nonimmunized Animals.* Ada has reviewed the experiments on the number of such cells that bind a specific ^{125}I-labeled protein or peptide.[7] Values of the order of 1/1000 or less are found. However, the usual experimental conditions (*eg* failure to saturate with antigen and counting of only "highly" labeled cells) are highly prejudiced toward low numbers (see below).

It is our intention to present some evidence that challenges the assumption of unispecificity in the precursor population, and to present some evidence for lack of unispecificity.

General Methodology

Our studies have been performed with a system used to measure the binding of β-galactosidase to a variety of lymphoid cells. β-Galactosidase is a stable bacterial enzyme with a mol wt of 540,000 and a high turnover number. This enzyme has been shown to be fully active when combined with antibody raised against it in rabbits.[8] Teased bone marrow, spleen, thymus or lymph node cells are washed and incubated with β-galactosidase in an ice bath for an hour. Alternatively, cells are fixed in the cold with 1% glutaraldehyde before they are incubated with enzyme. The cells are washed extensively to dispose of unbound antigen and then a variety of developing substrates can be added. Using the technic of Rotman,[9] who was able to measure the product of one enzyme molecule, we have employed fluorescein-di-β-galactoside as a

substrate. Free fluorescein released into microdroplets or in tubes can be accurately measured. Alternatively, and for most of the work to be reported here, an indigogenic substrate, 5-bromo-4-chloro-3–indolyl-β-D-galactoside (BIG) [10] is added and a blue precipitate is deposited in the vicinity of the enzymatic reaction. A recent clever alternative devised by Rotman and Cox [11] is the use of riboflavin galactoside as substrate, which when cleaved to give free riboflavin will feed an auxotrophic mutant that can grow up into a visible colony. In each of these methods, advantage is taken of the repeated utilization of the bound enzyme antigen in continuing to churn out product, thus greatly amplifying the sensitivity of detection of each cell-bound molecule. These methods can be used to study binding by any of the numerous determinants on the β-galactosidase molecule.

Certain mutant enzymes (AMEF) can be reactivated by combination with antibody directed against a determinant on active β-galactosidase. Rotman and Celada [12] have developed a modification of the fluorogenic assay that uses AMEF; this should reduce any background due to uptake by nonantibody sources and should obviate the need to remove excess antigen, thus allowing measurement of antigen-receptor binding under equilibrium conditions. However, Rotman and Modabber [13] have found that AMEF is not activated by cell receptor antibody whereas free antibody does activate AMEF. Other mutant enzymes [14] perhaps will prove useful for studying cell-bound receptors. A further modification can be used to detect antihapten antibodies on cells, if the hapten-galactosidase is exposed to cells.[15]

Inhibition of binding of the active enzyme can be accomplished by treating the initial cell suspension with cross-reactive enzymatically inactive protein, anti-immunoglobulins or proteolytic enzymes.

Results

Number of Receptors per Cell

Using the fluorogenic method, we have been able to determine the number of β-galactosidase molecules bound on individual mouse thymus or marrow cells.[16,17] We found from 30,000 to almost 1 million molecules on single active cells, with about fourfold larger numbers on marrow cells. Cell-containing droplets showing early fluorescence were selected by eye from a large network. It is, therefore, a selected population having a higher mean activity than the true average. It is of interest that there is more than a 50-fold range in the number of molecules bound per cell, suggesting a broad cellular heterogeneity in receptor

content. Differences in receptor affinity no longer apply after the cell is distributed in its microdroplet: if enzyme molecules detach from the cell surface, they nevertheless will be counted. Despite the high density of receptor on the most active antigen-binding cells, a large proportion of their surface is still available to house H-2, θ and other surface antigens of lymphocytes.

Frequency of Antigen-Binding Cells

It is also possible to use Poisson analysis to determine the frequency of antigen-binding cells by the fluorogenic microdrop method, but this is somewhat cumbersome, especially if it is of interest to determine the relatively low as well as high binders. Therefore, we have made use of the chromogenic "BIG" method, which gives the same values for the frequency of β-galactosidase binders. The BIG method may also give an indication of the localization of receptors on individual galactosidase-binding cells.

Some typical fixed mouse spleen cells incubated with β-galactosidase and then with BIG are shown in Fig 1 A–D. In Fig 1A, is a survey field illustrating the clear distinction between BIG-positive and -negative cells. Cells with color only slightly above background make up a very minor part of the population. Most positive cells are the size of small to medium lymphocytes (Fig 1A, B and C) though larger cells are seen occasionally (Fig 1D). Most positive cells have a patchy appearance and a variety of patterns have been observed (Fig 1A–D). The average frequencies found for mouse and rabbit thymus, marrow and spleen are shown in Table 1.

Table 1—β-Galactosidase-Binding Cells in Unimmunized Animals

	Antigen-binding cells/10^3 cells		
Animal	Thymus	Marrow	Spleen
A/Jax mice	1.4	44.0	18.9
Rabbits	0.6	11.4	2.6

As has been reported previously,[16,17] we find a sizable number of binders in mouse thymus, attesting to the relative sensitivity of the chromogenic method. That these cells are immunologically relevant is suggested by preliminary experiments using cortisone. Blomgren and Andersson [18] and Cohen and Claman [19] have shown that within 48 hours after mice are injected with about 2.5 mg of cortisone, a 95% loss of cells from the thymus occurs. The remaining cell population

appears to include all of the immunologically relevant cells as measured by GVH initiation in a recipient, and restoration of the response to sheep red blood cells in an irradiated, thymectomized recipient. Table 2 shows the dramatic increase in the proportion of galactosidase-binding cells after cortisone treatment.

Table 2—Effect of Cortisone on Antigen-Binding Cells in Mouse Thymus

Treatment	Antigen-binding cells/10^3 cells		
	Exp 1	Exp 2	Exp 3
Normal thymus	1.0	0.8	0.5
Thymus, 44 hours cortisone after	11.8	3.9	12.0

If these values of several percent of galactosidase-binding cells are not artifactual and not unique for this particular antigen (or a small number of antigens), then it becomes very unlikely that hypotheses postulating single specificities on antigen-binding cells in unimmunized animals can remain tenable.

Test of Possible Artifacts

We have carefully considered possible artifacts.[20] Saturation curves with increasing galactosidase concentration have been determined with various cell suspensions. For example, with mouse bone marrow cells, the same number of binding cells is obtained for a tenfold concentration range (10–100 μg/ml of β-galactosidase) if the cells treated with the lower concentrations of enzyme are incubated long enough with BIG. At 1 μg/ml, the induction period is greatly extended and a lower limiting value is reached. At still lower concentrations, no galactosidase-binding cells could be detected. Rabbit marrow cells behave somewhat differently than mouse marrow cells in that saturation is reached at lower concentrations and, on the average, more galactosidase is bound per cell. No change in the number of binders is seen over a 1000-fold range.

Size measurements, combined with acridine orange staining, indicated that a majority of the bone marrow antigen-binding cells were small or medium lymphocytes.

Similarly, incubating cells at 37 C with glass beads to remove macrophage-like cells did not selectively remove a large proportion of galactosidase-binding cells. Procedures designed to remove cytophilic antibody (as conventionally defined) also had no effect.

We have also considered the possibility that some cells become labeled by secondary capture of escaped reaction product. Two types of experiments were done to rule out this possibility. First, it was shown that cells not incubated with galactosidase do not become blue when suspended in the blue supernatant from a BIG reaction mixture. Second, when the reaction was carried out in many microdroplets (each containing approximately 25 cells), the predicted statistical distribution, as well as the same total proportion, of active cells was found as when the reaction was run in a test tube.

To test the possibility that some galactosidase binding was occurring through interaction with a substrate-like molecule on the cell surface, cells and β-galactosidase were incubated alone or in the presence of lactose or isopropylthiogalactoside. No difference in the number of BIG-positive cells was found.

Most importantly, we have shown that the binding of galactosidase to cells of the mouse lymphon is strongly inhibited when the cells are preincubated with some rabbit anti–mouse immunoglobulins. Under carefully controlled conditions,[20] it has been found that more than 90% of the putative BIG-positive cells preincubated with these specific antisera develop blue color far more slowly than control cells preincubated with normal rabbit globulin.

Therefore, by all the criteria usually used for antigen-binding studies, there is no reason to doubt the immunologic relevance or specificity of the response.

We also considered the possibility that β-galactosidase is an unusual antigen in that, through some accident of intrinsic antigenic stimulation, it was bound by a large number of cells. We have measured the number of galactosidase binders in tissues of neonatal rabbits and found a larger frequency in the thymus than is found in the adult[21] (Table 3). Our best evidence against external stimulation comes from a collaborative study with Y. B. Kim in which galactosidase-binding cells were measured in germfree, colostrum-deprived piglets[22] (Table 3). It can be seen that these animals already have rather high

Table 3—Binding Cells in Neonatal Animals

Animal	Antigen-binding cells/10^3 cells		
	Thymus	Marrow	Spleen
Germfree colostrum-deprived piglets	4.0	13.6	2.2
Neonatal rabbits	2.6	3.3	3.1

numbers of binding cells prior to full term. It is clear that in a case where intrinsic infection can be ruled out, or in fact any circulating immunoglobulin, there can be a very high proportion of binding cells.

Binding of Horseradish Peroxidase

The *ad hoc* argument that, for some freakish reason, a large number of receptors to galactosidase naturally exists in the mouse and the pig becomes quite improbable as an increasing number of cases of high-proportion antigen-binding cells are reported. Indeed, we have searched for other enzymes for which high values obtain. Horseradish peroxidase seems to be such an enzyme. We have found that, after treatment of lymphoid cell populations with hydrazine to destroy the rather considerable intrinsic peroxidase activity,[23] it is possible to measure the binding of horseradish peroxidase by adding 3-amino-9-ethyl carbazole (CAR) and hydrogen peroxide.[24] A highly localized, red reaction product results (Fig 1E and F). The values obtained for mouse (and rabbit) tissues are of the same order as those obtained for galactosidase binding. Furthermore, peroxidase binding was as inhibited by rabbit anti-mouse κ-chain immunoglobulin as was galactosidase binding (Table 4).

Table 4—Inhibition of Mouse Marrow Binding with Rabbit Anti-Mouse κ - Chain Globulin

Enzyme	Antigen-binding cells/10^3 cells	
	Normal rabbit globulin	Anti-κ globulin
β-Galactosidase	74.0	13.0
Horseradish peroxidase	20.0	4.4

Peroxidase and Galactosidase Binding in the Same Cell Population

We next investigated the summation of galactosidase and perovidase binding. Cells were first incubated with β-galactosidase, then BIG and then hydrazine to destroy intrinsic peroxidase, then with horseradish peroxidase and finally CAR + peroxide. The results of one such experiment, as well as the single controls, are indicated in Table 5. It can be seen that the values for BIG and CAR are the same in the double-incubation experiment as the values determined separately. The appearance of two cells in the same preparation—one positive for galactosidase and the other for peroxidase—is shown in Fig 1G.

This result indicated that there was no gross overlap of peroxidase

Table 5—Independence of Antigen BInding by Individual Antigen-Binding Cells

Antigen exposure	Galactosidase-binding cells/10^3 cells	Peroxidase-binding cells/10^3 cells
Thymus		
β-Galactosidase	2.2	
Horseradish peroxidase		4.2
β-Galactosidase + Horseradish peroxidase	1.5	2.9
Marrow		
β-Galactosidase	49	
Horseradish peroxidase		26
β-Galactosidase + Horseradish peroxidase	56	18
Spleen		
β-Galactosidase	23	
Horseradish peroxidase		44
β-Galactosidase + Horseradish peroxidase	24	71

and galactosidase binders, and it greatly encouraged us to search for direct proof that lymphoid cells from unimmunized animals have antigen-binding cells of multiple specificity. In its simplest form, a pluripotential model with random assortment of specificities on different binding cells would predict that double binders occur at a frequency equal to the product of the frequencies of the two single-binding cells. In addition, if doubles could be found with a separate distribution of peroxidase and of β-galactosidase on a single cell, one form of pluripotentiality might be ruled out. In a recent review,[2] Mäkelä and Cross have suggested a pluripotential model which results from single primordial binding cells, each having a unique receptor but of rather broad specificity.

In our first experiments, we have indeed found cells that appear to be double galactosidase-peroxidase binders. They appear to have separate sectors for binding of each enzyme rather than admixed binding. In fact, most single binding cells also give the impression of being patchily stained. Two of these cells are shown in Fig 1H and I.

The frequency of doubles that we find is also roughly consistent with our simple hypothesis. We must emphasize that these results are based on examination of very few doubles and considerably more extensive studies are now in progress.[25] In addition, we have preliminary evidence for the occurrence of doubles at about the expected frequency in two other systems: the galactosidase–sheep red cell rosette system and the galactosidase–glucose oxidase couple. Since we see

rosettes and glucose oxidase cells at a much lower frequency than BIG or CAR cells, there is a considerable experimental difficulty in amassing observations on large or even moderate numbers of doubles.

Discussion

High Values in Studies by Others

Our value of greater than 1% antigen-binding cells for a single antigen in normal mouse marrow and spleen is considerably higher than most but *not all* previously published frequencies for antigen-binding cells in unimmunized animals. Most workers using ^{125}I-proteins and radioautography have mainly concentrated on the most highly labeled cells and have reported values of 0.2–2 antigen binders per 1000 nucleated cells.

It is a general criticism of most published results that cells have been incubated with far less than saturating amounts of labeled antigen. For example, in an experiment in which about 0.15 ^{125}I-flagellin binders per 1000 mouse spleen lymphocytes were found, prior or simultaneous addition of a 1000-fold excess of unlabeled flagellin did not reduce this value, clearly indicating that very few of the available sites had been occupied with ^{125}I-flagellin. On the other hand, increasing the amount of ^{125}I-flagellin causes a striking increase in the number of antigen-binding cells, as can be observed in the curves of Byrt and Ada.[26]

Dwyer and Mackay[27] have shown that 2% of the cells in human thymus bind ^{125}I-flagellin when the cells are exposed to 2.5 mg flagellin/ml. They showed that binding was specific by demonstrating strict additivity for binding when a second non-cross-reactive flagellin was used singly and in combination. Nevertheless, they concluded that flagellin binding must be an unusual case involving an extraordinarily high number of receptor cells.

Ada and co-workers found a frequency of at least 1.6% for hemocyanin binders when they raised the incubation concentration to a saturating value.[28] They also found that 2% of mouse peritoneal cells bind hemocyanin.[26]

We would reiterate that Dwyer and Mackay found a high number of specific binders in a fetal organ, the thymus from a 20 to 24-week-old human fetus. Similarly, Dwyer and Warner[29] have recently reported that about 0.8% of the bursal cells from a 16-day-old chicken embryo bind ^{125}I-flagellin.

These results should be considered together with the work of Simon-

sen, who found 2% of lymphocytes could engage in a GVH reaction [30] and the results of Wilson and co-workers, who found 3% of an unprimed population could engage in a mixed lymphocyte reaction.[31]

Lack of Allelic and Class Restriction

It would be instructive at this point to review the evidence regarding restriction in producer and precursor cells. There is now quite an accumulation of evidence supporting the idea that antibody-producing cells in the mouse, rabbit and human show allelic exclusion and class exclusion, as well as a restriction in specificity.

This has been found with immunoglobulin-secreting myelomas, antibody-secreting cells and plasma cells of normal animals, although some exceptions have been reported. This work has recently been reviewed by Mäkelä and Cross.[2]

The situation with respect to precursor cells is not as clear. We would like to review several recent cases in which an apparent lack of allotypic or class exclusion has been observed with precursor cells.

1. The indication in the rabbit allotype work of Sell, Lowe and Gell [32] is that the blast transformation they observe upon adding a mixture of two specific antiallotype sera to heterozygous rabbit cells is *more* than can be accounted for by summing the blastogenic effect obtained by adding the sera singly. This suggests cooperative effects resulting from the presence of both allotypic specificities on the surface of the cells stimulated.

2. In the work of H. Anderson,[33] the conclusion was drawn that mouse spleen cells are nonallelically excluded with respect to IgG_{2a} prior to their differentiation into antibody-secreting cells. Irradiated mice bearing the Ig-1^a allele were repopulated with Ig-$1^{a/b}$ heterozygous cells, which were injected together with sheep red cells. At various times after this, anti-Ig-1^b serum was injected, which might have been expected to obliterate the response of the Ig-1^b spleen cells. In fact, the Ig-1^a responses were also drastically reduced, suggesting that most cells expressed both a and b determinants. If several days had elapsed after the sheep red cell injection before the anti-Ig-1^b serum was given, some restriction had already occurred because now the Ig-1^a response could partially occur.

3. The third case is that of Greaves' study of rosette inhibition. He looked at mouse Ig-$1^{a/b}$ heterozygote rosette-forming cells during the anti–sheep red cell response. Soon after antigen injection, adding either anti-Ig-1^a or anti-Ig-1^b serum could prevent a certain percentage of rosettes from forming. He expected summation of the inhibitory effects

when both specific sera were employed but in fact found that the observed inhibition was much lower than that expected.[34] Similar experiments demonstrated a lack of class restricton in mice [35] as had been previously demonstrated in guinea pigs.[36] In both the allotypic and class restriction experiments, early in the response there appears to be little restriction, but by 2 weeks to 1 month after immunization, additivity of inhibition by each antiserum in the mixture is established, indicating restriction.

4. The final example concerns the lack of allelic exclusion in normal As 4/6 rabbits.[37] In these recent studies with the sensitive "mixed antiglobulin reaction," [38] 50–75% of rosette-forming cells gave evidence of bearing both the As4 and As6 allotype on their surface. Interestingly, the large majority of these mixed rosettes were "balanced," having an equal distribution of each of the allotypic determinants, although up to a third of the mixed rosettes clearly were "unbalanced" and had a preponderance of one or the other specificities. The use of many ingenious controls in these studies excluded the possibility of artifact due to passive absorption. However, two other groups, who used immunofluorescence or autoradiographic technics and somewhat different cell preparations, found few [39] or no [40] rabbit peripheral lymphocytes showing lack of allelic exclusion.

The case is strong (though not airtight) for a lack of allotypic or class restriction on the globulin-bearing precursor cells of the mouse and rabbit. The evidence of Anderson and Greaves supports the notion of restriction occurring at some time after antigen injection, which would then result in the unispecificity of antibody-producing cells. Regarding the antibody-forming cell precursors, however, there is little reason to expect restriction of specificity if there is indeed a lack of allelic exclusion. Although the existence of a single functional V-gene per cell and multiple C-genes is surely possible, it seems more likely that, on cells bearing receptors derived from two different chromosomes, there would be at least two different V-genes.

Conclusion

Our results, in which we find large numbers of specific antigen-binding cells with little overlap of specificity, clearly suggest that most of these cells that can be shown to bind two antigens appear to bind these antigens at independent sites. This last result requires some substantiation but does seem to rule out the model proposed by Mäkelä and Cross [1] in which pluripotentiality of any single cell would result from a single "primordial" receptor having a broad specificity. Instead, our

data support a model in which pluripotentiality results from the presence of two or more specificity types of receptor on single cells.

The demonstration from several laboratories that allelic exclusion does not appear to occur until *after* contact with antigen removes one of the major props used to support the idea that any one antigen-binding cell must contain only a single species of receptor molecules. On the other hand, our results, as well as those demonstrating lack of allelic exclusion, do not rule out the presence of subpopulations of antigen-binding cells which do, indeed, have a unique receptor. Furthermore, the possibility still exists that the pluripotential capacity of antigen-binding cells may not be a heritable property of these cells; *eg*, multiple receptors may be acquired by some process such as reversible membrane fusion between cells.

Although not unequivocally established, if heritable pluripotentiality is assumed, then a model is suggested which may provide a framework for further experiments. The primary cell that reacts with antigen is considered to be one that has developed (or acquired) the capacity to make several immunoglobulin sequences. The coding for these sequences may be redundant and extrachromosomal, and subject to unequal partition in daughter cells. Contact with antigen causes blastogenic stimulation. Division with unequal partition of information and continued selection for a particular specificity leads to the development of memory without depletion of the specificity of the lymphocyte pool for other antigens. That is, individual antigen-binding cells still contain a random assortment of specificities except that there is now a prejudice for more cells to contain the specificities that correspond to the administered antigen. Furthermore, as the antigen concentration drops, selection becomes increasingly stringent, resulting in antibody-producing cells of a single particular specificity, and therefore, of a single class and allotype.

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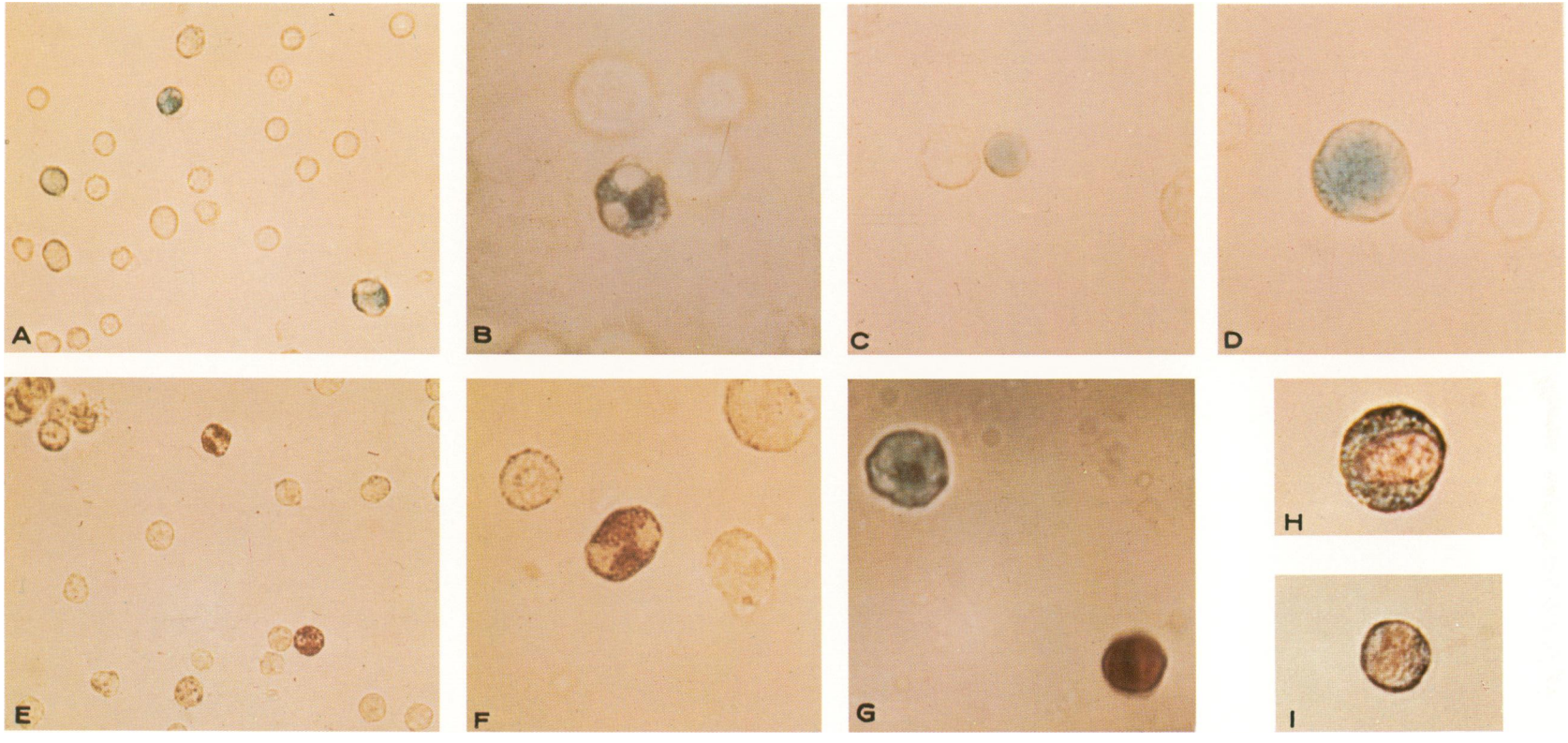

Fig 1—Mouse spleen cells showing specific binding of β-galactosidase, horseradish peroxidase or both. **A–D**—Cells showing β-galactosidase binding as indicated by enzymatic production of indigo dye. **E and F**—Cells showing horseradish peroxidase binding as indicated by enzymatic production of peroxidation product of 3-amino-9-ethyl carbazole. **G–I**—Cells first treated with galactosidase and its chromogenic substrate, then with hydrazine, and finally with peroxidase and its chromogenic substrate. **G**—Cells independently staining for galactosidase and peroxidase. **H and I**—Cells showing double staining indicating the presence of both galactosidase and peroxidase (A and E, × 430; B–D, F, G, × 1080).