Bacterial Adherence to Eucaryotic Cells: Isolation of Lymphocyte-Binding Mutants

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A procedure for obtaining bacterial mutants that bind to eucaryotic cells is described. This procedure takes advantage of the ability of the mutants to obtain a required nutrient from the eucaryotic cells. We used this procedure to isolate mutants of *Escherichia coli* that bind to mouse lymphocytes. We show that the mutants identify some immunoglobulin-bearing lymphocytes and some non-immunoglobulin-bearing lymphocytes.

Bacterial adherence to epithelial cell surfaces has been used to study host-parasite interactions for many years (3-5, 10, 13, 14). However, it was only recently that bacterial adherence to cell surfaces has been used to identify subpopulations of morphologically indistinguishable cells (7, 15, 18), to isolate and functionally characterize cell subpopulations (6), and to distinguish between normal and abnormal cell populations (17).

Thus far, the identification and characterization of subpopulations of cells by their bacterial binding properties have been applied to mouse (7) and human (18) lymphocyte populations. However, bacteria are known to bind to many kinds of eucaryotic cells (1-3, 5, 12, 13). Thus, the binding of bacteria by eucaryotic cells may become the basis of general methods for identifying and characterizing subpopulations of morphologically indistinguishable cells and for detecting abnormalities in cell populations.

The usefulness of bacterial adherence as a means of studying cell populations would be greatly increased if it were possible in laboratories to select for bacterial mutants that bind to the cells of interest. This would simplify the task of obtaining the appropriate bacteria and perhaps result in the identification of cell subpopulations that could not be detected by the use of naturally occurring strains of bacteria. The availability of genetically defined strains of bacteria that differ only in their ability to bind to particular cells would also facilitate the elucidation of the mechanisms by which bacteria bind to cells.

In this paper, we describe a selection procedure that can be used to isolate bacterial mutants that bind to animal cells. We used this procedure to isolate lymphocyte-binding mutants of *Escherichia coli*. We show that the mutants differ from the parent strain in their

ability to bind to a subpopulation of mouse lymphocytes.

MATERIALS AND METHODS

Preparation of spleen cell suspensions. BALB/c mouse spleen cell suspensions were prepared as described previously (7).

Preparation of bacterial cell suspensions. The bacteria were grown at 37°C in antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) for 24 to 48 h with continuous aeration. The cells were harvested by centrifugation at $8,000 \times g$ for 5 min, washed with saline, and suspended in 10% formaldehyde in calcium- and magnesium-free Dulbecco phosphate-buffered saline (GIBCO Laboratories, Grand Island, N.Y.). After 18 to 24 h at 4°C, the cells were harvested, washed four times with saline, and resuspended in saline to a density of approximately 10^{10} cells per ml.

Bacterial adherence assay. Lymphocytes in Eagle minimal essential medium (GIBCO Laboratories) supplemented with 6% bovine serum albumin and 0.02% sodium azide were mixed with bacteria at a ratio of at least 200 formaldehyde-fixed bacteria for each lymphocyte in a volume of about 0.1 ml. The cells were centrifuged together at $900 \times g$ at 4°C for 5 min to promote binding, and the pellet was suspended by pipetting. The cells with bacteria attached were scored by phase-contrast microscopy.

Mutagenesis of bacteria. A log-phase culture of *E. coli* YS57 (*pro his trp*) was harvested, washed twice with 0.1 M citrate buffer (pH 5.5), and suspended in citrate buffer to a density of 5×10^8 cells per ml. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma Chemical Co., St. Louis, Mo.) was added to give a final concentration of 50 µg/ml, and the bacteria were incubated for 30 min at 37°C. The bacteria were harvested by centrifugation, washed once with 0.1 M phosphate buffer (pH 7.0), and inoculated into Davis minimal medium (Difco Laboratories) supplemented with 20 µg each of proline, histidine, and tryptophan per ml. The surviving cells were grown at 37°C with aeration.

Preparation of Ig⁻ lymphocytes. Mouse immunoglobulin-bearing lymphocytes (Ig⁺ cells) were rosetted with sheep erythrocytes coated with purified rabbit anti-mouse immunoglobulin G (IgG) antibodies (16) by

mixing the antibodies with the lymphocytes at a ratio of 100:1 and centrifuging the cells together at $900 \times g$ for 5 min at 4°C to promote binding. The rosetted Ig^+ cells were separated from the unrosetted non-immunoglobulin-bearing lymphocytes (Ig^- cells) by centrifugation on Ficoll-Hypaque medium at $400 \times g$ for 20 min at 4°C (16). The unrosetted Ig^- cells were collected from the top of the Ficoll-Hypaque layers and washed twice with Eagle minimal essential medium (GIBCO Laboratories).

RESULTS AND DISCUSSION

Isolation of lymphocyte-binding mutants. We employed a proline-, histidine-, and tryptophan-requiring strain of *E. coli*, YS57, that did not bind naturally to mouse spleen cells. The rationale of the selection procedure was that lymphocyte-binding mutants of YS57 would be able to grow in the absence of exogenously supplied tryptophan by obtaining tryptophan from the lymphocytes to which they were bound, whereas the bacteria that were not bound to the lymphocytes would not grow. Since YS57 was Trp^- as a result of a deletion of the genes for the tryptophan biosynthetic enzymes, the problem of selecting Trp^+ revertants during the procedure was eliminated. YS57 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and the bacteria that survived were grown in medium containing proline, histidine, and tryptophan. To enrich for lymphocyte-binding mutants, the bacteria were mixed with mouse spleen cells at a ratio of 100 bacteria to 1 spleen cell, and the cells were pelleted by centrifugation to promote the attachment of potential lymphocyte-binding mutants. After incubation for 1 h at 4°C , the pellet was suspended, and the mixture was gently layered on 5 ml of saline containing 6% (wt/vol) bovine serum albumin. The cells were centrifuged at $150 \times g$ for 10 min, and the supernatant containing the unbound bacteria was discarded. The pellet containing the lymphocytes and any bacteria associated with them was inoculated into Davis minimal medium containing proline, histidine, and tryptophan, and the bacteria were grown overnight at 37°C with continuous aeration. Although this enrichment procedure was repeated six times, no enrichment in lymphocyte-binding mutants was detected by the bacterial adherence assay. Therefore, after the sixth round of enrichment, instead of inoculating the cells into growth medium, we added approximately 5×10^6 lymphocytes and any bacteria associated with them to 2.5 ml of tryptophan-free medium supplemented with proline, histidine, and agar (0.3%). The soft agar was then poured on minimal agar plates (1.5% agar) supplemented with proline and histidine, and the plates were incubated at

37°C . Under these conditions, the bacteria could grow only if they obtained tryptophan from the lymphocytes. Thus, only those bacteria that bound to the lymphocytes or those that were near the lymphocytes in the soft agar layer were expected to grow. After 4 days at 37°C , microcolonies were visible. The plates were overlaid with soft agar containing tryptophan and then incubated overnight at 37°C . After incubation, the plates contained several large colonies, which arose from the microcolonies, and many small colonies, which probably arose from the unbound bacteria in the lymphocyte suspension. The large colonies from several mutant hunts were picked and tested for their ability to bind to mouse lymphocytes and for their amino acid requirements. Approximately 5% of the large colonies represented lymphocyte-binding mutants. Three independent mutants (UI2010, UI2022, and UI2023), all of which required proline, histidine, and tryptophan, were selected for further studies. Each of these mutants came from a different mutant hunt.

We have not determined whether it is absolutely necessary to incorporate the enrichment procedure in the mutant hunts. It may be possible to apply the selection procedure to tryptophan-free medium without first applying the enrichment procedure. However, since the enrichment procedure should increase the proportion of mutants in the population of bacteria, we routinely incorporated it into our mutant hunts.

Other methods for obtaining lymphocyte-binding mutants can undoubtedly be devised. For example, it should be possible to obtain mutants by simply applying the enrichment procedure many times. How many times the enrichment procedure would have to be applied is not known. Obviously more than six rounds of enrichment would be required since we were unable to detect any lymphocyte-binding mutants after six rounds of enrichment. The advantage of the present method is that there is a strong selection procedure for the mutants. Thus, the time required for obtaining the lymphocyte-binding mutants is reduced.

Binding of the mutants to mouse spleen cells. The fraction of the spleen cell population that was labeled by each of the mutants was determined by mixing the mutants with spleen cells and counting the number of spleen cells with bacteria attached. On the average, all of the mutants bound to more than 25% of the spleen cell population, whereas the parental strain, YS57, only bound to about 1% of the population (Table 1). These data show that our selection procedure could be used to obtain bacterial mutants that identify a subpopulation of the mouse spleen cells.

To determine whether the mutants bound to Ig⁺ cells or to Ig⁻ cells, we compared the percentages of cells with bacteria attached when the mutants were tested on spleen cell populations (about 50% Ig⁻ cells) with the values obtained when they were tested on populations enriched in Ig⁻ cells (<90% Ig⁻ cells). The Ig⁻ cells prepared in this way were not pure T cells, but they were enriched in T cells. The fraction of the Ig⁻ cell population labeled by the mutants was essentially equal to or slightly less than the fraction of the spleen cell population labeled, which indicated that the enrichment in Ig⁻ cells did not result in a concomitant increase in the percentage of labeled cells (Table 2). Thus, it is likely that the mutants identify some Ig⁻ cells (presumably T cells) and some Ig⁺ cells (B cells).

In this study, we employed *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as a mutagen and made use of the tryptophan requirement of YS57 as the basis of the selection for lymphocyte-binding mutants. It may be possible to select for spontaneous mutants or for mutants induced by other mutagens. In addition, it should be possible to use other nutritional requirements as the basis of the selection procedure, as long as the lymphocyte can supply the required nutrient. Thus, many different mutagens and bacterial strains may be useful for selecting lymphocyte-binding bacterial mutants. This may result in the isolation of a number of different lympho-

cyte-binding mutants that can be used to characterize lymphocyte subpopulations.

The mechanism by which the mutants bind to the lymphocytes is not known. However, since the mutants bind to a large fraction of the lymphocyte population, it is unlikely that the binding is mediated by immune receptors. Indeed, preliminary data suggest that the binding is not mediated by immune receptors or by passively adsorbed antibacterial antibodies on the lymphocytes. We are currently investigating whether the binding is mediated by lectins or by bacterial pili. Both pili and lectins have been implicated in the binding of bacteria to cells (2, 8, 9, 11).

The availability of lymphocyte-binding bacterial mutants and the nonbinding parent strain from which they were derived provides a new way of probing the surface components of lymphocytes and should facilitate the characterization of lymphocyte subpopulations and the elucidation of the mechanisms by which the mutants bind to the lymphocytes. The mechanisms by which lymphocytes and bacteria interact may help us to understand how lymphocytes interact with other lymphocytes or with other cells in the production of an immune response. Finally, since the selection procedure for obtaining lymphocyte-binding bacterial mutants does not depend upon a unique property of the lymphocytes, the selection procedure should be useful

TABLE 1. Binding of mutants UI2010, UI2022, and UI2023 to BALB/c splenic lymphocytes

Bacteria	% of lymphocytes with bacteria attached in expt ^a :						Mean (%)
	1	2	3	4	5	6	
YS57	1 ± 0.3	1 ± 0.3	1 ± 0.3	1 ± 0.3	1 ± 0.3	1 ± 0.3	1
UI2010	39 ± 1.6	31 ± 1.2	29 ± 1.2	30 ± 0.9	20 ± 1.5	21 ± 1.5	28
UI2022	19 ± 0.3	ND ^b	32 ± 0.6	32 ± 1.1	ND	26 ± 3.5	27
UI2023	33 ± 1.6	30 ± 1.8	ND	32 ± 1.1	27 ± 1.5	32 ± 1.5	31

^a Mean value of triplicate counts of 100 cells ± the standard error.

^b ND, Not done.

TABLE 2. Binding of UI2010, UI2022, and UI2023 to Ig⁻ cell populations

Bacteria	% of population with bacteria attached in expt ^a :						Mean (%) ^b	
	1		2		3		Spleen cells	Ig ⁻ cells
	Spleen cells ^c	Ig ⁻ cells ^d	Spleen cells	Ig ⁻ cells	Spleen cells	Ig ⁻ cells		
UI2010	21 ± 1.0	17 ± 1.0	19 ± 1.5	18 ± 1.0	22 ± 1.5	14 ± 1.5	21	16
UI2022	26 ± 3.5	15 ± 1.5	21 ± 1.5	15 ± 1.0	22 ± 0.5	15 ± 2.5	23	15
UI2023	32 ± 1.5	16 ± 1.0	25 ± 1.0	17 ± 1.5	24 ± 2.5	16 ± 1.0	27	16

^a Mean values of triplicate counts of 100 cells ± the standard error.

^b Mean value of the individual experiments.

^c Percentages of Ig⁺ cells in the spleen cell population were 47, 46, and 44 for experiments 1, 2, and 3, respectively.

^d Percentages of contaminating Ig⁺ cells in the Ig⁻ cell population were 4, 9, and 9 for experiments 1, 2, and 3, respectively.

in obtaining bacterial mutants that bind to any eucaryotic cell. Thus, bacterial adherence to eucaryotic cells may become a widely used tool for studying subpopulations of morphologically indistinguishable cells.

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