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Method for identifying microbial antigens that stimulate specific lymphocyte responses: Application to *Salmonella*

(λgt11/expression library/T-cell epitopes/mouse immunity)

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ABSTRACT **Vaccine development and understanding of cellular immune stimulatory mechanisms have been impeded by the paucity of data on microbial antigens that stimulate protective immunity. We describe here a general method for identifying and isolating peptide antigens that specifically stimulate sensitized lymphocytes. First, *Salmonella typhimurium* C5 genomic DNA fragments were subcloned into *Escherichia coli* by use of the λgt11 expression vector. Next, antigens expressed by recombinant phage from this genomic library were tested for their capacity to stimulate proliferative responses in pooled lymphocytes obtained from BALB/c mice infected 14 days earlier with *S. typhimurium*. Of 2000 recombinant phages tested, 5 stimulated a polypeptide-antigen-specific proliferative response. Physical analyses of these 5 recombinant phages revealed cloned inserts of 0.5–2.4 kilobase pairs representing nonoverlapping regions of the C5 chromosome. Four of the five insert DNAs hybridized at high stringency to both *S. typhimurium* and *Salmonella typhi* total chromosomal DNA, suggesting that these pathogens of different host specificity share several antigenic determinants. Use of sensitized primary polyclonal lymphocytes provides a rapid and simple method for screening recombinant DNA libraries for clones that stimulate specific immune responses and avoids the use of cloned lymphocyte cell lines. This approach should be generally applicable to similar studies in different hosts of many other microbial pathogens.**

Salmonella typhimurium causes a systemic disease in mice that has been used as a model to understand the human enteric fever process caused by *Salmonella typhi*. In animal studies with *S. typhimurium*, live attenuated vaccines have been reported to provide superior protection when compared with killed whole-cell or subcellular vaccines (1–3). The effectiveness of live *Salmonella* vaccines has been attributed to the survival of the attenuated strain in the host long enough to induce a protective cellular immune response, in addition to a humoral response. Live vaccines typically stimulate long-term protection against disease, but the antigens involved in stimulating protective immunity remain largely undefined. Indeed, the relative importance of humoral versus cellular immunity in protection against *Salmonella* infections remains controversial (4).

In earlier studies, Paul *et al.* (5, 6) showed that adoptive transfer of antigen-specific T-cell lines to BALB/c mice, which are *ity*^{S} (i.e., hypersusceptible to *S. typhimurium*), provides significant protection against a challenge with virulent *S. typhimurium* SR11. These T-cell lines, isolated from BALB/c mice that had been sensitized with a protein-rich extract of *S. typhimurium* were subsequently expanded with this protein-rich mixture. Although these results suggest that cellular immunity directed against protein antigens plays a pivotal role in protective immunity in BALB/c mice, the specific *Salmonella* peptides involved in stimulating these T cells remain uncharacterized.

We describe here a combined molecular cloning and immunological screening strategy designed to identify *Salmonella* polypeptide antigens recognized by primary polyclonal sensitized lymphocytes. Five recombinant phages, the lysates of which stimulate a polypeptide-antigen-specific proliferative response by lymphocytes from infected BALB/c mice, have been isolated from a λgt11 expression library of the *S. typhimurium* C5 genome and preliminarily characterized. Recombinant clones expressing *Salmonella* antigens can now be used to expand antigen-specific T- or B-cell lines. This approach, which employs polyclonal lymphocytes obtained from infected animals of choice, has general applicability for the specific identification, from many pathogens, of key immunogenic determinants involved in stimulating host-protective immune responses.

MATERIALS AND METHODS

Bacterial Strains. *S. typhimurium* C5 and SR11 are fully virulent strains (intraperitoneal LD_{50} of <10 cells), each of which harbors a 100-kilobase-pair (kb) virulence-associated plasmid. Strain WS1321 is a plasmid-cured isogenic derivative of strain SR11 (7). *S. typhi* Ty2 is a classical virulent human typhoid bacillus. *Escherichia coli* Y1090 served as the recipient of the λgt11 phage recombinants (8). All strains were grown at 37°C with constant aeration in NZCYM broth (Difco) or on NZCYM agar plates supplemented with ampicillin (100 μg/ml). Expression of cloned inserts was induced by incubating phage-infected *E. coli* at 42°C in NZCYM broth supplemented with 1 mM isopropyl β-D-thiogalactopyranoside.

Construction of a λgt11 Expression Library. A *Hin*fI λgt11 expression library was constructed by the method of Stover *et al.* (9). Total genomic DNA isolated from *S. typhimurium* C5 was treated with RNase, extracted with phenol/chloroform, 1:1 (vol/vol), and then dialyzed against TE buffer (50 mM Tris·HCl, pH 7.5/1 mM EDTA), as described by Maniatis *et al.* (10). Genomic *Salmonella* DNA (10 μg) was partially digested in the presence of 0.05 unit of *Hin*fI (New England Biolabs) for 30 min at 37°C. Digested DNA was extracted once with phenol and once with phenol/chloroform, 1:1 (vol/vol), and then precipitated with ethanol (10). The average molecular size of the partial digest was determined to be 3 kb by agarose gel electrophoresis. Twenty micromoles of unphosphorylated *Eco*RI–*Hin*fI adaptors (9) was ligated to 2 μg of limit-digested *Hin*fI *Salmonella* DNA fragments. The DNA ligase was removed by extracting the ligation mixture with phenol/chloroform/isoamyl alcohol, 24:23:1 (vol/vol), and then with chloroform/isoamyl alcohol, 23:1 (vol/vol). Excess adaptors were removed by selective precipitation of the high molecular

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weight DNA with PEG, as described by Stover *et al.* (9). The *Eco*RI adaptor-linked genomic DNA was phosphorylated with T4 polynucleotide kinase (10) and then ligated with 0.5 unit of T4 DNA ligase to 1 μg of dephosphorylated *Eco*RI-digested λgt11 DNA. These adaptors, oligonucleotide mixtures of 13, 14, and 15 base pairs, were designed so that genomic fragments are inserted into the λgt11 *Eco*RI cloning site in all three possible translational reading frames. This ligand mixture was then packaged with a commercially available λ DNA packaging system (Promega). The *in vitro*-packaged DNA was used to infect *E. coli* Y1090. The resulting library had 1×10^6 phage per ml, 70% with inserts, as determined on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) medium (10).

Lymphocyte Proliferation Assay. The lymphocyte proliferation assay was based on a modification of a method described by Corradin *et al.* (11). Six- to 8-week-old female BALB/c mice, purchased from Harlan (Indianapolis, IN), were caged in groups of five. The mice were sensitized by subcutaneous injection of 1×10^4 cells of *S. typhimurium* WS1321. Fourteen days after infection, the mice were sacrificed by cervical dislocation, and the periaortic and inguinal lymph nodes were pooled immediately. A single-cell suspension was prepared and plated in 96-well microculture plates as described by Paul *et al.* (5). Recombinant antigen was added at a protein concentration of 2 μg per well. The plates were incubated for 48 hr at 37°C with 5% CO_2/95% air. Then, 1 μCi of [*methyl*-^{3}H]thymidine (1 Ci = 37 GBq) was added and the cells were incubated for an additional 18–24 hr. The cells were harvested with a semiautomated PHD cell harvester (Cambridge Technology, Cambridge, MA). The amount of [^{3}H]thymidine incorporated was determined by liquid scintillation counting. The mean and standard deviation of the results of three to six replicate wells are presented. Significant differences in proliferative responses of the lymphocytes were determined using an analysis of variance with a random block design.

To determine optimum lymphocyte concentration, 2 μg of recombinant lysate protein was added to lymphocytes ranging from 4×10^4 to 4×10^5 cells per 200 μl per well. Under these conditions, the optimum number of lymphocytes per assay was 8×10^4 for all recombinant lysates except for Sty88, which was 4×10^4 (see Fig. 1*A*). Later assays were conducted with the optimal lymphocyte number to obtain maximal proliferative responses.

Preparation of Recombinant Antigens. Two-thousand independently isolated 5-bromo-4-chloro-3-indolyl β-D-galactoside-negative plaques were picked with sterile 100-μl micropipets. The agar plugs for 3 to 5 plaques were transferred to 1 ml of isopropyl β-D-thiogalactopyranoside-supplemented NZCYM that had been inoculated with 0.5 ml of an overnight culture of Y1090. These cultures were incubated for 3 hr at 42°C, and then bacterial cells were lysed by adding one drop of chloroform. The cell debris was removed by centrifugation for 5 min in a Microfuge and a 100-μl sample containing recombinant λ phage was stored at 4°C. The protein content of the λgt11 lysate was measured by the Bio-Rad protein assay (Bio-Rad) and then 2 μg of protein per well was added to the lymphocytes. Each antigen was added to three to six replicate wells and assayed. Recombinant lysates that stimulated a positive proliferative response were plaque-purified. Lysates from purified plaques were rescreened with a different batch of sensitized lymphocytes.

Proteinase K Digestion of Recombinant Antigens. Lysates from Y1090 cells infected with lymphocyte-stimulatory recombinant phages as well as the cloning vector λgt11 were digested with proteinase K (50 μg/ml in 0.5% SDS) at 65°C for 1 hr. Since SDS is toxic to lymphocytes, we spotted 20 μl of the proteinase K-digested lysates and an equivalent amount of each undigested lysate (treated with 0.5% SDS) onto 5 × 5 mm squares cut from sterile BA85 nitrocellulose paper (Schleicher & Schuell; the total protein content spotted was 20 μg). The filter squares were washed extensively with RPMI 1640 medium supplemented with gentamicin (50 μg/ml). The antigen on these nitrocellulose squares was processed for presentation to lymphocytes by dissolving the nitrocellulose squares with dimethyl sulfoxide, precipitating as a fine particulate suspension in 0.1 M sodium carbonate (pH 9.8), washing twice with RPMI 1640 medium supple-

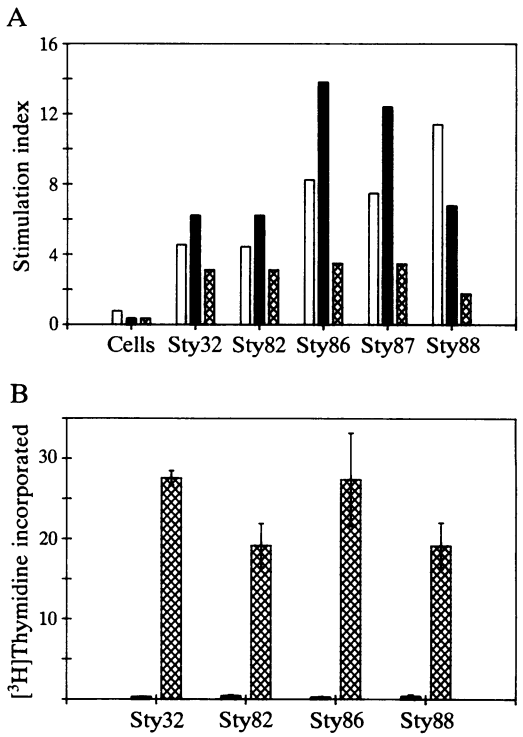

FIG. 1. Proliferative response of lymphocytes to untreated and proteinase K-treated lysates of recombinant phage expressing *Salmonella* proteins. Regional lymph nodes were isolated from five BALB/c mice infected 14 days earlier by subcutaneous injection of 1×10^4 cells of *S. typhimurium* WS1321. A single-cell suspension of the lymph nodes was prepared and these lymphocytes were treated with antigen. (*A*) Response of lymphocytes to recombinant λgt11 phage lysates. Lymphocytes were treated with 2 μg of protein from control λgt11 lysates or lysate protein from each recombinant λgt11 phage Sty32, Sty82, Sty86, Sty87, or Sty88. The data are expressed as stimulation indexes [mean [^{3}H]thymidine (cpm) incorporated by lymphocytes from six replica assay wells treated with recombinant lysate/mean [^{3}H]thymidine (cpm) incorporated by lymphocytes from six replica assay wells treated with λgt11 lysate]. The open, solid, and cross-hatched bars represent the stimulation index obtained with 4×10^4, 8×10^4, and 4×10^5 lymphocytes per well, respectively. Note that the untreated lymphocyte controls (i.e., cells) had stimulation indexes of <1. The mean lymphocyte proliferative response to recombinant phage lysates was compared with that of wild-type λgt11 lysate by analysis of variance using a random block design ($P = 0.01$). None of the numerous other tested recombinant phage lysates stimulated a proliferative response that was significantly higher than lymphocytes treated with λgt11 lysate. (*B*) Proliferation of sensitized lymphocytes to proteinase K-digested recombinant lysates. Samples (40-μg) of phage protein lysate from λgt11 Sty32, Sty82, Sty86, and Sty88 were digested with 100 μg of proteinase K for 1 hr at 65°C and then 20-μl samples were spotted on nitrocellulose and processed to remove SDS. Positive control lysate samples were processed with SDS but proteinase K was omitted. Samples of nitrocellulose-bound proteinase K-digested (solid bar) and control (cross-hatched bar) antigens were added to 1×10^5 sensitized lymphocytes and the proliferation assay carried out. The data are expressed as cpm ($\times 10^{-3}$) (mean ± standard deviation) of six replica assay wells. Although not shown, Sty87 lysate stimulated lymphocyte proliferation and was also sensitive to proteinase K digestion.

mented with gentamicin (50 μg/ml), and then resuspending the pellet in 1 ml of RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, gentamicin (50 μg/ml), 50 μM 1-mercaptoethanol, and 2 mM L-glutamine (12).

Southern Blot Analysis. Restriction endonuclease-digested *Salmonella* DNA and recombinant phage DNA were separated by electrophoresis through a 0.7% agarose gel in TBE buffer (0.050 M Tris base/0.050 M boric acid/0.001 M EDTA). DNA was transferred to nitrocellulose, as described by Maniatis *et al.* (10).

Hybridizations were conducted overnight at 65°C in an aqueous solution containing 6× SSC (20× SSC = 3 M NaCl/sodium citrate, pH 7.0), 5× Denhardt's solution (100× Denhardt's solution = 10 g of polyvinylpyrrolidone/10 g of bovine serum albumin/10 g of Ficoll 400/H_2O to 500 ml), 1% SDS, and salmon sperm DNA (100 μg/ml). Probes were labeled with [^{32}P]dCTP by nick-translation (New England Nuclear). The filters were washed at 65°C with 2× SSC/0.1% SDS, then with 1× SSC/0.1% SDS, and then with 0.1× SSC/0.1% SDS.

Plasmid Isolation. The 90-kb virulence plasmid was isolated from *S. typhimurium* C5 by the alkaline lysis method of Kado and Liu (13). The plasmid DNA was purified by CsCl/ethidium bromide density gradient centrifugation, followed by dialysis against TE buffer.

Polymerase Chain Reaction. Insert DNA was amplified from recombinant phage by polymerase chain reaction (14). Five microliters of phage stock (10^9 plaque-forming units/ml) was diluted into 45 μl of distilled water, boiled for 4 min, and then rapidly cooled on ice. To 0.5 μl of target phage DNA, λgt11 primer DNA (100 pmol of λgt11 F 5'-GCATGGATCCGGTGGCGACGACTCCTGGAGCCCG-3', λgt11 R 5'-GCATAAGCTTTGGTAATGGTAGCGACCGGCGC-3'), 200 μM dATP, 200 μM dGTP, 200 μM dTTP, and 100 μM dCTP, and 1 unit of AmpliTaq polymerase (Perkin–Elmer/Cetus) were mixed in reaction buffer (25 mM Taps·HCl, pH 9.3/50 mM KCl/2 mM $MgCl_2$/1 mM 2-mercaptoethanol; Taps is *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid). The reaction mix was placed in a DNA thermal cycler (Perkin–Elmer/Cetus) and was heated to 95°C for 5 min, then 30 cycles of reannealing at 65°C for 1 min, DNA synthesis at 72°C for 1 min, and denaturation at 95°C for 1 min. Each reaction product (2 μl) was analyzed by agarose gel electrophoresis (1% agarose in TBE buffer).

RESULTS

Expression Library Construction. An expression library of *S. typhimurium* C5 genomic DNA was constructed by cloning partially *Hin*fI-digested bacterial DNA into the *Eco*RI site of λgt11 with *Hin*fI/*Eco*RI adaptors. These adaptors were designed to provide ligated products in all three translational reading frames. This library contained 1 × 10^6 phage per ml, with 70% of the phage carrying insert DNA.

Identification of Cloned Lymphocyte-Stimulatory Antigens. Recombinant phage expressing immune-reactive *S. typhimurium* antigens were detected by a lymphocyte-proliferation assay. Lymphocytes were prepared as a pooled single-cell suspension of periaortic and inguinal lymph nodes from female BALB/c mice infected 14 days earlier by subcutaneous injection of viable cells of *S. typhimurium* WS1321. These lymphocytes were treated with 2 μg of protein from an isopropyl β-D-thiogalactopyranoside-induced phage lysate mixture prepared by infecting *E. coli* Y1090 with three to five insert-positive (i.e., Gal$^-$) plaques. Phage from lysates producing stimulation indexes of 2 or more (Fig. 1) were plaque-purified and retested for their capacity to stimulate a proliferative response. From a total of about 2000 recombinant phages tested, 5 plaque-purified recombinant phages were found to be immunoreactive (Fig. 1*A*) and were further characterized.

Definition of the Peptide Nature of Stimulatory Antigens. To determine if the five cloned *Salmonella* inserts were expressing polypeptides that were recognized by the sensitized polyclonal lymphocyte pools, we digested the five recombinant lysates with proteinase K. SDS present in the proteinase digestion reaction is toxic to lymphocytes and was removed by first binding the proteinase K-treated samples and untreated samples supplemented with 0.5% SDS to 5 × 5 mm nitrocellulose squares. Then, the bound antigen was washed, solublized, precipitated, and presented to pooled lymphocytes. As shown in Fig. 1*B*, proteinase K digestion of all recombinant phage lysates reduced proliferation to the same level as untreated control lymphocytes (i.e., lymphocytes treated only with nitrocellulose). These results demonstrate that the lymphocyte-stimulatory material in the recombinant lysates was polypeptide in nature. Also, the small amount of lipopolysaccharide in each lysate did not stimulate a significant mitogenic response because, under these experimental conditions, lipopolysaccharide from *S. typhimurium* and *E. coli* binds to nitrocellulose and will stimulate a proliferative response by lymphocytes if present at 1–100 μg (data not shown).

Five Recombinant Phages Stimulate Antigen-Specific Nonmitogenic Lymphocyte Proliferation. Because porins (15) and lipoprotein (16) can sometimes act as nonspecific mitogens, recombinant phages expressing these peptides should stimulate a nonspecific proliferative response by naive lymphocytes. Table 1 shows that phage lysates prepared from *E. coli* Y1090 infected with the cloning vector λgt11 stimulated a 6.7- to 9.2-fold increased proliferation when compared with untreated naive lymphocytes. In contrast, none of the five recombinant phage lysates studied above stimulated a proliferative response above the untreated control lymphocytes. The proliferation stimulated by the recombinant lysates was significantly (P = 0.05) lower than that of lymphocytes treated with the same amount of λgt11 lysate. This is in contrast to the response of lymphocytes obtained from *Salmonella*-sensitized mice (Fig. 1). The mechanism by which

Table 1. Proliferative responses of lymphocytes from naive BALB/c mice to recombinant phages

	[^{3}H]Thymidine incorporation, cpm					
Protein, μg	λgt11	Sty32	Sty82	Sty86	Sty87	Sty88
0.0	374 + 189	514 + 183	487 + 331	400 + 184	285 + 232	741 + 257
0.12	306 + 87	414 + 200	270 + 124	353 + 166	229 + 8	487 + 241
0.25	2521 + 305	412 + 200	210 + 70	436 + 246	448 + 183	682 + 217
0.50	2881 + 752	290 + 130	173 + 33	306 + 85	337 + 119	588 + 188
1.00	2936 + 298	746 + 233	380 + 269	489 + 267	399 + 126	493 + 145
2.00	3459 + 916	691 + 195	411 + 76	544 + 142	351 + 61	504 + 197

A single-cell suspension was prepared from the mesenteric lymph nodes of naive BALB/c mice, as described by Paul *et al.* (5). Approximately 1 × 10^5 lymphocytes were treated with protein (0.0–2.0 μg/ml) from recombinant phage lysates. [^{3}H]Thymidine incorporation is presented as the mean plus standard deviation.

the recombinant lysates depress the proliferative response relative to control λgt11 lysates is unknown.

Physical Analysis of Five Recombinant Insert DNAs. All five recombinant phage DNAs hybridized to *S. typhimurium* C5 chromosomal DNA and not to the 90-kb virulence-associated plasmid carried by this strain (data not shown). Digestion of the λgt11 recombinant phage DNAs with *Eco*RI released a 2.5-kb insert fragment from λgt11 Sty32, whereas no insert DNA was released from the other four recombinant phages. These four recombinant phages apparently had lost one or more of their *Eco*RI sites, but each carried a unique insert DNA as determined by multiple restriction endonuclease digestions with five endonucleases (data not shown). To determine the approximate insert size of all five recombinant phage, the insert DNA was amplified by polymerase chain reaction with oligonucleotide primers that flanked the *Eco*RI site of λgt11. Fig. 2 shows that insert DNAs of about 2.5, 1.3, 0.48, 0.8, and 1.0 kb were recovered for recombinant phages Sty32, Sty82, Sty86, Sty87, and Sty88, respectively. When the amplified DNAs were used to probe restriction endonuclease digests of *S. typhimurium* C5 and *S. typhi* Ty2 DNA, the probes Sty32, Sty82, Sty87, and Sty88 hybridized to unique DNA fragments, generally of similar size from both bacterial species (Table 2). In contrast, phage Sty86 hybridized only to *S. typhimurium* DNA. None of the Sty probes cross-hybridized to one another. Thus with restriction fragment analysis data, this information strongly suggests that each clone represents a unique *Salmonella* chromosomal DNA region.

DISCUSSION

There is a paucity of information about microbial antigens that stimulate a host-protective cellular immune response and the mechanisms involved in generation of this response. This is due largely to the unwieldiness of available methods of identifying antigens that stimulate a specific T-cell response. In earlier attempts to identify cloned *Salmonella* gene products involved in triggering a cellular immune response, genomic libraries in pBR322 and λgt11 were first screened by immunoblot methods with *E. coli*-adsorbed *Salmonella*-specific polyclonal sera that was obtained from infected rabbits or mice. However, in an examination of >5000 cloned *Salmonella* genomic segments, none of the immunoblot-positive clones stimulated an antigen-specific proliferation of sensitized pooled lymphocytes (data not shown). This failure led us to search for a more direct screening method to detect antigens that specifically stimulate T lymphocytes. Rather than using the limited and more time-consuming approach of isolating monoclonal antibodies or cloned T-cell lines to select undefined antigens of interest, we combined expression cloning techniques to isolate *Salmonella* antigens with the use of lymphocyte proliferation assays employing sensitized pooled lymphocytes to identify five chromosomally encoded *Salmonella* peptides that stimulate a specific lymphoproliferative response. Although these primary lymphocyte cultures contained sensitized B and T cells, the proliferation of the sensitized B cells is dependent upon the presence of sensitized T cells (17) and measures antigen-specific T-lymphocyte responses. The size and chromosomal location of the genes encoding these antigens, the phenotype of the responding lymphocytes, and whether the antigens stimulate a protective immune response should be investigated. Surprisingly, DNA of four of the five immunoreactive recombinant phages hybridized under conditions of high stringency with the *S. typhi* chromosome, which raises the possibility that these organisms share important antigens that may eventually have prophylactic and diagnostic clinical value.

Screening of cloned antigenic genes with lymphocytes from a sensitized animal of choice promises to have great value as a tool for identifying specific immune stimulatory peptides from many pathogens, whether bacterial, fungal, parasitic, or viral. This approach may not identify stimulatory

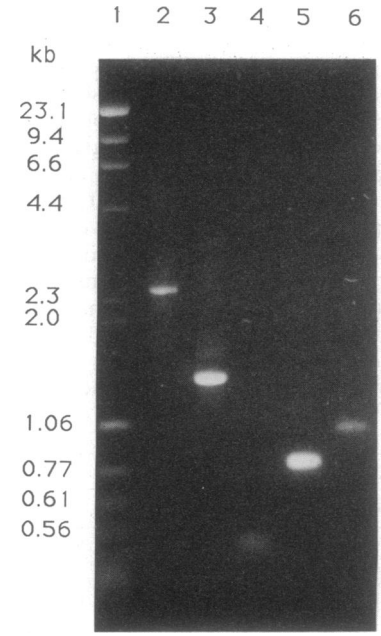

FIG. 2. Size of *Salmonella* DNA in recombinant clones. *Bst*EII λ-digested DNA standards (lane 1), λgt11 Sty32 (lane 2), Sty82 (lane 3), Sty86 (lane 4), Sty87 (lane 5), and Sty88 (lane 6) are shown. Insert DNA was amplified from recombinant phage by polymerase chain reaction.

Table 2. *S. typhimurium* C5 and *S. typhi* Ty2 genomic DNA fragments homologous to recombinant clones

Probe	Endonuclease	C5, kb	Ty2, kb
Sty82	*Bam*HI	4.6	4.6
	*Eco*RI	>20	>20
	*Hin*dIII	>20	5.9
	Pst I	9.9	9.5
Sty86	*Bam*HI	9.6	ND
	*Eco*RI	>20	ND
	*Hin*dIII	11.2	ND
	Pst I	18.7	ND
Sty87	*Bam*HI	17.8	15.5
	*Eco*RI	4.8	4.7
	*Hin*dIII	10.0	9.1
	Pst I	0.7	ND
Sty88	*Bam*HI	>20	>20
	*Eco*RI	14.8	14.3
	*Hin*dIII	>20	>20
	Pst I	>20	>20
Sty32	*Bam*HI	>20	>20
	*Eco*RI	14.8	14.3
	*Hin*dIII	>20	>20
	Pst I	>20	>20

C5 or Ty2 DNA was digested with *Bam*HI, *Eco*RI, *Hin*dIII, or *Pst* I for 1 hr at 37°C. The DNA fragments were separated on 0.7% agarose in TBE buffer. The DNA was transferred to a nylon membrane, cross-linked by UV irradiation (120,000 μJ), and probed with random-primer-labeled insert DNA. The probe DNA was hybridized to the target DNA at 65°C for 18 hr. The filter was washed successively with 2× SSC, 1× SSC, and 0.1× SSC/0.1% SDS at 65°C. The filters were stripped with 10 mM pyrophosphate buffer (pH 6.5) in 50% (vol/vol) formamide for 1 hr at 65°C and then washed with 2× SSC (10). The filter was then probed with another recombinant clone. ND, none detected.

epitopes formed by the interaction of nonadjacent peptides, peptides requiring unique membrane structures for proper processing and presentation, or complex carbohydrate antigens such as lipopolysaccharide. Despite these potential limitations, this general system for the identification of specific immune stimulatory peptides will be useful for developing vaccines that super-stimulate protective immunity or for constructing chimeric T-cell-reactive fusion peptides that stimulate immune responses, as reported by Etlinger *et al.* (18).

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