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Production of Potent *Salmonella* H Antisera by Immunization with Polymeric Flagellins

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Highly purified polymeric flagellin preparations from 10 different *Salmonella* serotypes were used to produce specific *Salmonella* H antisera with high titers by the immunization of rabbits. Antigen emulsions in complete Freund adjuvant were administered at the rate of 50 μg per rabbit by multiple intradermal injection. Booster injections were given 110 days after the primary immunization. The immune response was monitored regularly over a period of 200 days. The results showed that the H titers, determined with ^{125}I-labeled antigens, averaged 61,000 ± 39,000, and the H agglutination titers of 83% of the animals were >40,000. The high titers of the immunized animals persisted for approximately 4 months. The O agglutination titers of the antisera were <10 for 9 of the flagellin preparations and ranged from 10 to 320 for the remaining preparation. The antisera obtained were serotype specific after appropriate dilution.

The production of flagellar (H) antisera for *Salmonella* serotyping is usually done by the intravenous injection of rabbits with live or formalinized broth cultures of highly motile *Salmonella* organisms at intervals of several days (8). This procedure gives sera containing not only flagellar antibodies but also somatic (O) antibodies. The H antisera titer in this case is frequently low, and the O antisera titer is often so high that it interferes with H agglutination results. Consequently, the O antisera are usually removed by absorption.

Alternatively, H antisera can be produced by the direct immunization of rabbits with flagellin preparations. Various workers have produced H antisera for different reasons by using this approach (4, 9–14). The protocols adopted, however, varied considerably in terms of amounts of immunogen used, immunization site or sites, and immunization and boosting schedules. Moreover, in most cases the characteristics of the antisera produced, such as titers and levels of contaminating O antibodies, have not been reported.

The production of antisera with high titers and specificity to *Salmonella* flagellins is of current interest (2, 6). This is because of the need to develop rapid immunoassay procedures for the detection of salmonellae in food and clinical specimens.

This communication reports a method for the production of potent *Salmonella* H antisera. The method has distinct advantages in that the immunized animals produce antisera with high H titers for a long time. Also, such antisera can be used directly without O antisera absorption for either *Salmonella* serotyping or other scientific investigations which require immunoassays (e.g., immunoradiometric assays, radioimmunoassays, and enzyme-linked immunosorbent assays).

MATERIALS AND METHODS

Organisms. Ten *Salmonella* serotypes were obtained from the Salmonella Reference Laboratory, Adelaide, South Australia. Reference number, H antigen type, and H antigen phase of each serotype are listed in Table 1. All serotypes were monophasic, except for *Salmonella kentucky* and *Salmonella worthington*, and in these cases, the unlisted H phases (i.e., i and z, respectively) were eliminated by immobilization with homologous *Salmonella* H agglutinating serum (Wellcome Diagnostics Dartford, England).

Antigens. Polymeric flagellins from each serotype were isolated in a highly purified form as described by Ibrahim et al. (G. F. Ibrahim, G. H. Fleet, M. J. Lyons, and R. A. Walker, submitted for publication). Briefly, this involved growing salmonellae in a chemically defined medium (3) supplemented with 0.01% yeast extract and 0.025% each of the amino acids DL-tryptophane, L-histidine, L-proline, L-threonine, L-arginine, glycine, DL-α-alanine, and L-methionine. After centrifugation of cells, flagella were detached by exposure at pH 2 for 30 min at room temperature. The flagellin in the supernatant was then centrifuged at 100,000 × *g* for 1 h at 4°C. The pH of the supernatant was adjusted to 7.2 and ammonium sulfate was added to achieve two-thirds saturation (2.67 M). Polymerized flagellin was separated by centrifugation and purified further by dialysis in tubing which had a molecular-weight cut-off of 50,000 (Spectrum Medical Industries, Los Angeles, Calif.). The dialyzed flagellins were then lyophilized.

Animals. Female New Zealand White rabbits (2.5 to 3.0 kg) were used. They were arranged into 10 groups of three or four animals for each serotype. Fur was shaved from the back and proximal limbs of each rabbit before immunization.

Immunization. The multiple intradermal injection method described by Vaitukaitis et al. (15) was used as follows. Polymeric flagellin was dissolved in sterile normal saline, and one volume of this solution and two volumes of complete Freund adjuvant (Commonwealth Serum Laboratories, Melbourne, Australia) were drawn into a 20-ml sterile syringe (Terumo, Melbourne, Australia). The syringe outlet was connected to another syringe with sterile stainless steel tubing (33 by 6 mm, outer diameter), threaded internally at each end. Emulsion formation was effected by continually forcing the mixture from one syringe to another until a stable emulsion was obtained. The emulsion preparation was prepared under aseptic conditions. Each emulsion was then administered intradermally whereby each animal received,

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TABLE 1. *Salmonella* serotypes used for raising antisera

Serotype[a]	Reference no.[b]	H antigen type	Antigen phase
S. oranienburg	1254	m,t	I
S. enteritidis	1267	g,m	I
S. kentucky	1285	Z_6	II
S. waycross	1312	Z_4,Z_{23}	I
S. abortus-equi	1451	e,n,x	II
S. tennessee	1623	Z_{29}	I
4,12:d:-	1634	d	I
Untypable[c]	1635	1,2	II
S. worthington	1695	l,w	II
S. lille	1721	Z_{38}	I

[a] All serotypes were monophasic except for *S. kentucky* and *S. worthington*, and in these cases, the unlisted phases (i.e., i and z, respectively) were eliminated before flagellin isolation.
[b] Salmonella Reference Laboratory, Adelaide, South Australia.
[c] 1,4,5,12:-:1,2.

on day one, a total of 2.0 ml (50 μg of flagellin) which was injected into 50 to 70 sites on the back. Booster injections were given in the same way 110 days after the primary immunization, except that incomplete Freund adjuvant was used. The immune response was monitored with serum separated from test blood samples that were obtained from an ear vein every 10 to 20 days over a total period of 200 days.

Radiolabeling of flagellins. The polymeric flagellins were depolymerized into monomeric forms with HCl and then radiolabeled as follows. The flagellins were dissolved in distilled water at a rate of 2.5 mg/ml. From each solution, 20 μl were removed, mixed with 5 μl of 0.25 M HCl, and after 30 min at room temperature, neutralized by the addition of 5 μl of 0.25 M NaOH. Subsequently, 20 μl of 0.625 M phosphate buffer (PB; pH 7.4) was added and mixed, and 10 μl of this mixture (containing 10 μg of monomeric antigen) was used for radiolabeling. Radiolabeling was done with a modification of the chloramine-T method (5) as follows. Monomeric antigen (10 μl) was added to 5 μl (0.5 mCi) of carrier-free $Na^{125}I$ in NaOH (Radiochemical Centre, Amersham, England) in a 50-by-10-mm glass tube. Chloramine-T, 5 μg in 10 μl of 0.05 M PB (pH 7.4), was then added. After gentle shaking of the reaction tube for 30 s by hand, 4 μg of sodium meta-bisulfite in 10 μl of 0.05 M PB was added. This was followed immediately by the addition of 150 μg of potassium iodide in 150 μl of 0.05 M PB containing 0.25% bovine serum albumin. The contents of the reaction tubes were then transferred to a disposable column of Sephadex G-25 with a bed volume of 9 ml (Pharmacia Fine Chemicals, Uppsala, Sweden) previously equilibrated with 0.05 M PB containing 0.25% bovine serum albumin. The reaction tube was rinsed with another 150 μl of the above potassium iodide solution, and this was also transferred to the column. Elution was carried out to separate free ^{125}I from radiolabeled flagellin. Any traces of free ^{125}I remaining in the radiolabeled flagellins were removed by overnight dialysis against running tap water.

Microprecipitation of ^{125}I-labeled antigens. Microprecipitation of ^{125}I-labeled antigens was carried out to determine reciprocal titers of antisera (referred to hereafter as titers). The titers were established by determining the highest dilution of antisera which precipitated 50% of radioactivity associated with labeled antigens.

Titer assays of antisera were carried out in polystyrene tubes (10 by 77 mm; Kayline, Keswick, South Australia) containing 100 μl of buffer consisting of 0.05 M PB, 0.85% NaCl, and 1% bovine serum albumin. This buffer was also used to dilute antisera and ^{125}I-labeled antigens. A 100-μl amount of appropriate serial dilutions of antisera and 100 μl of ^{125}I-labeled antigen at 10,000 to 15,000 cpm were added to the tubes, and they were incubated for 2 h at 37°C with gentle orbital shaking for antigen-antibody reaction. Subsequently, 100 μl of cellulose suspension coated with anti-rabbit antibody raised in donkey (Sac-Cel; Wellcome Diagnostics) were added, and the mixture was incubated at room temperature for 30 min. The unbound radiolabeled antigen was eliminated by adding 3 ml of distilled water, followed by centrifugation at 1,700 × *g* for 5 min and aspiration. The residual radioactivities on the cellulose pellets were determined by using a Packard Selectronic Autogamma spectrometer. Dose-response curves were plotted after correction for the nonspecific binding of radiolabeled antigens, and the titers were determined.

Agglutination. H tube agglutination was done by incubating 0.5 ml of two-fold serial dilutions of antisera and 0.5 ml of an 18-h *Salmonella* culture in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) at 50°C for 2 h. Preparations of O antigens for the O tube agglutination titers were obtained by boiling washed *Salmonella* cells for 10 min to destroy H antigens. After washing of the boiled cells, 0.5-ml samples of cell suspensions were mixed with 0.5 ml of diluted antisera and incubated at 50°C for 4 h to determine O agglutination titers.

Slide agglutination tests were also performed in the standard way.

RESULTS

H titers with ^{125}I-labeled antigens. The radiolabeling method outlined above consistently produced labeled flagellins with specific activities of 30.2 ± 10.0 μCi/μg. The incorporation of radioactive iodine into the antigens averaged 72.6%, with a standard deviation of 14.4%.

The immune response of the animals measured by microprecipitation of the respective ^{125}I-labeled flagellin antigens varied considerably. As expected, such variations were observed not only between groups of animals but also within each group. The immune responses of six animals immunized with six different flagellin antigens are depicted in Fig. 1. The response of these six animals represented a cross-section of the response of the 10 groups of animals. Low titer levels were observed 30 days after the primary immunization, and these levels accelerated thereafter. Significant decreases in titers were not observed 102 days after the time of the primary immunization. However, we decided to rechallenge the animals with a booster injection. The effect of such boosting was not clear. A response to boosting seemed to occur with animals immunized with antigens e,n,x, l,w, and 1,2, whereas animals immunized with antigens Z_6, d, and Z_{29} did not appear to respond to boosting (Fig. 1). Generally, antisera titers of virtually all animals peaked approximately 150 days after the primary immunization and averaged 61,000 ± 39,000.

Tube agglutination of H antisera. High levels of H agglutination titers were generally detected 30 days after primary immunization. These levels, however, varied considerably during the postimmunization period. During this period, the titers ranged from 2,500 to 1,280,00 (Fig. 2 and 3). The effect of booster injections 110 days after the primary immunization appeared to produce an increase in the antiserum for the animal immunized with antigen Z_{29}. However, this increase may have been a coincidental change which was not related

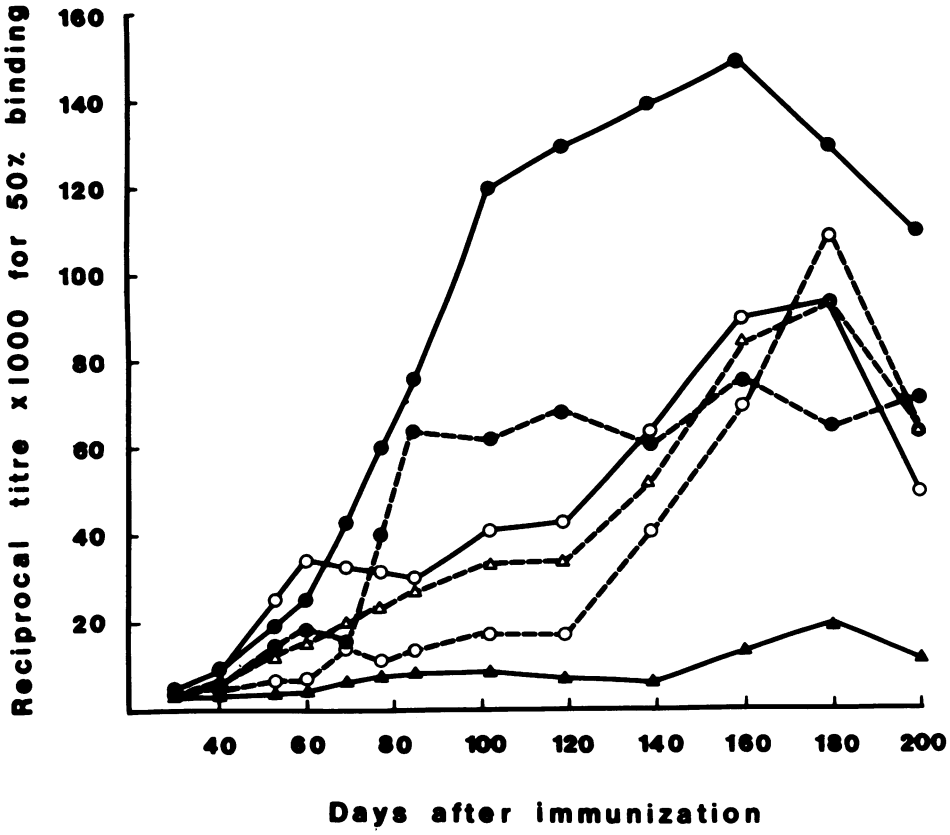

FIG. 1. Reciprocal titers of antisera from six rabbits during the period of immunization. Data are expressed as the reciprocal of the antiserum dilution required to bind 50% of homologous ^{125}I-labeled flagellin. Boosting was done 110 days after primary immunization. The H antigens used for immunization were (●——●) Z_6, (●- - -●) d, (○——○) l,w, (○- - -○) 1,2, (▲——▲) Z_{29}, and (△- - -△) e,n,x.

to boosting. Antiserum from the animal immunized with antigen l,w showed an increase in titer. However, this occurred 30 days after the booster injection. Had this increase been due to boosting, then it should have occurred approximately 14 days after boosting. Moreover, the increase in titer was modest when compared to the titer of antiserum 60 days after the primary immunization (Fig. 3). Apart from the antiserum from the animal immunized with

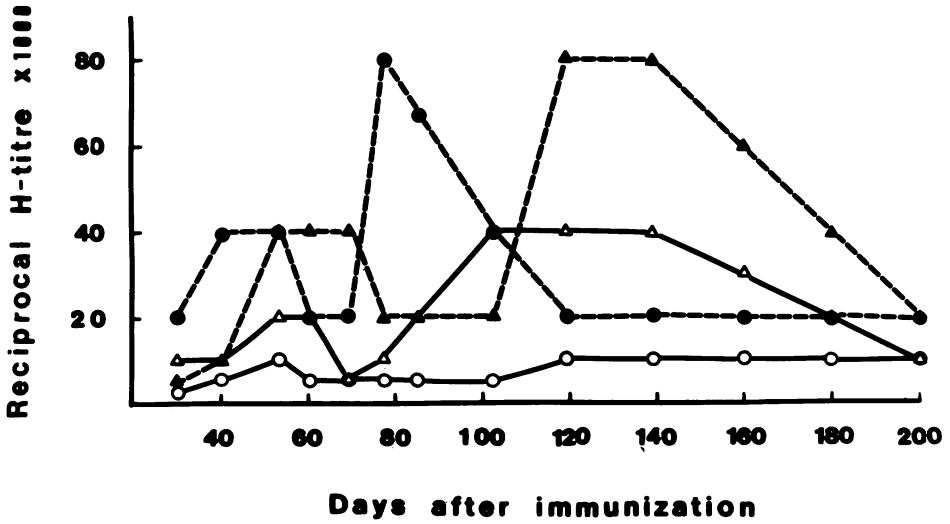

FIG. 2. Reciprocal H agglutination titers of four rabbits during the period of immunization. Boosting was done 110 days after primary immunization. The H antigens used for immuniation were (●) e,n,x, (○) 1,2, (▲) d, and (△) Z_6. The above antisera were obtained from the same rabbits as those shown in Fig. 1.

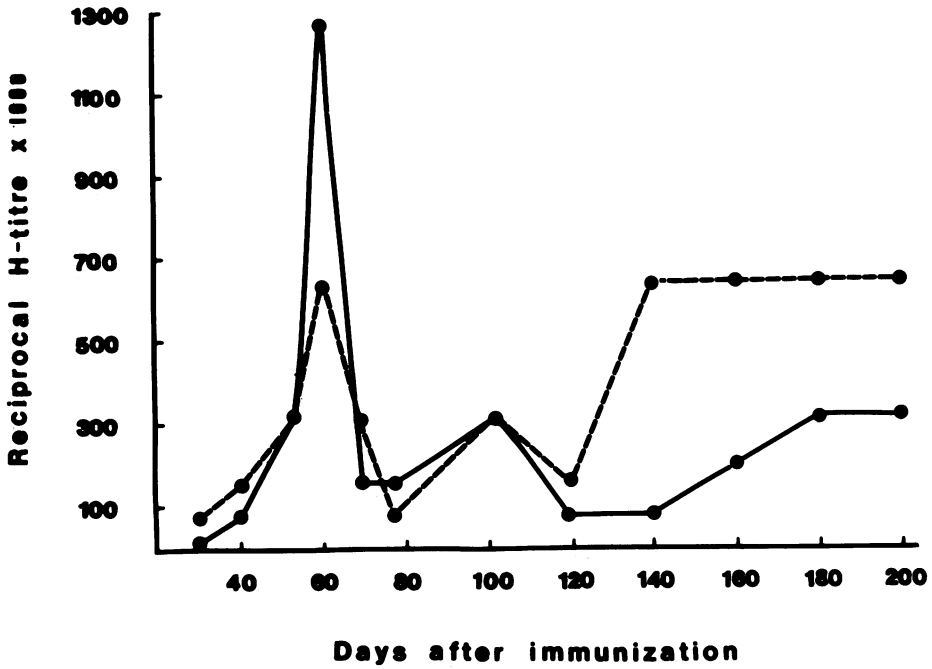

FIG. 3. Reciprocal H agglutination titers of two rabbits during the period of immunization. Boosting was done 110 days after primary immunization. The H antigens used for immunization were (●——●) l,w and (●- - -●) Z_{29}. The above antisera were obtained from the same rabbits as those shown in Fig. 1.

antigen d, the boosting process clearly had no effect on the H titers of antisera from animals immunized with the antigens Z_6, e,n,x, or 1,2 (Fig. 2).

The distribution frequency of H titers for the 36 immunized rabbits is shown in Table 2; the majority of the animals produced titers of 40,000 to 80,000.

Tube agglutination of O antisera. The titer of O antisera, which was regularly monitored during the entire postimmunization period (200 days), was <10 with antisera from nine groups of animals immunized with nine different immunogens. Antisera of the remaining group, which was immunized with flagellin from *Salmonella waycross*, had an O titer in the range of 10 to 320. Boosting appeared to have no effect on such titers.

Specificity of antisera. Specificity of antisera was determined with the tube agglutination test. A high degree of specificity was found, as the antisera obtained with any given immunogen could only agglutinate the *Salmonella* serotype from which such immunogen was isolated. No cross-agglutinations were observed with the tube agglutination test, even when the antisera were used at a dilution of 1:500.

However, cross-reactions were observed between certain antisera and *Salmonella* serotypes when the slide agglutination test was performed with undiluted antisera, due to the presence of common antigenic determinants on *Salmonella* flagellins (G. F. Ibrahim, G. H. Fleet, M. J. Lyons, and R. A. Walker, Med. Microbiol. Immunol., in press). The results (Table 3) showed that every individual antiserum agglutinated (apart from its homologous serotype) one to five heterologous serotypes. However, the agglutination titers of homologous serotypes were at least fourfold the titers of heterologous serotypes. The dilution of such antisera (1:2,000) rendered them serotype specific with the slide agglutination test.

TABLE 2. Distribution frequency of peak *Salmonella* H agglutination titers among 36 rabbits immunized with H antigens

H antigen type	No. of animals with H titer ($\times 10^3$) of:								
	5	10	20	40	80	160	320	640	1,280
m,t				1	2				
g,m	1			2	1				
Z_6			1	1	2				
Z_4,Z_{23}					1	1		2	
e,n,x		1		2					
Z_{29}				1	2		1		
d					3				
1,2		2		2					
l,w							1		2
Z_{38}		1			3				

DISCUSSION

The multiple intradermal injection method (15) was described for the production of specific antisera for hormone haptens. We have shown, using a modification of this method, that it is possible to produce antisera with high titers for protein antigens in polymeric form, i.e., flagellin preparations from *Salmonella* species. This method has the following advantages: (i) the titer remains high for approximately 4 months, thus allowing the collection of large amounts of antisera without sacrificing the animals; (ii) the O antisera titers are low, and thus, the H antisera do not require absorption; (iii) H antigens of salmonellae can be serotyped with confidence, using either the tube or the slide tests, after appropriate dilution of such antisera; and (iv) only small amounts of flagellins (50 μg per rabbit) are required for immunization.

Generally, the results of this study compare favorably with an earlier study (4), in which a higher level of *Salmonella* flagellins was used for immunization, lower H antisera

TABLE 3. Cross-reactions between *Salmonella* serotypes and homologous and heterologous antisera as determined by slide agglutination

H antigen used for antisera production	Highest agglutination titers of H antigens of *Salmonella* serotypes:									
	m,t	g,m	Z_6	Z_4,Z_{23}	e,n,x	Z_{29}	d	1,2	l,w	Z_{38}
m,t	**4,000**	500	—[a]	—	500	—	—	—	—	—
g,m	1,000	**8,000**	—	—	—	—	—	—	—	—
Z_6	—	—	**4,000**	—	—	—	1,000	500	500	500
Z_4,Z_{23}	—	—	—	**2,000**	500	500	—	—	—	500
e,n,x	—	—	—	—	**2,000**	—	500	500	—	—
Z_{29}	—	—	—	500	—	**2,000**	—	—	—	500
d	—	—	1,000	—	500	—	**4,000**	500	500	500
1,2	—	—	500	—	500	—	500	**8,000**	500	—
l,w	—	500	—	500	—	500	500	500	**8,000**	—
Z_{38}	—	—	—	—	500	500	—	—	—	**2,000**

[a] —, No agglutination occurred even when undiluted antisera were used.

titers were obtained, and such antisera contained higher levels of contaminating O antisera (titers up to 1,280).

The antigen emulsions used by Vaitukaitis et al. (15) for the production of antisera for hormone haptens contained a final concentration of 2.5 mg of heat-killed *Mycobacterium tuberculosis* per ml, and each animal was administered antigen emulsion as well as 0.5 ml of crude *Bordetella pertussis* vaccine, presumably to enhance the immune response to the antigens. In the current investigation, the final concentration of heat-killed *M. tuberculosis* in antigen emulsions used for the primary immunization was only 0.3 mg/ml, and the use of *B. pertussis* vaccine was omitted. This was decided because *Salmonella* flagellins are known to be highly immunogenic (1, 12), and the unnecessary administration of bacterial antigens other than *Salmonella* flagellins to the rabbits was considered undesirable.

Although, in producing this high titer H antisera we boosted the animals 110 days after the primary immunization, the value of such boosting was questionable. It is conceivable that boosting was ineffective because it was done at a time when there was no significant decrease in antisera titers.

Titer determinations were carried out with the microprecipitation of ^{125}I-labeled antigens. The radiolabeling conditions, described in the present investigation, were designed to produce labels with low specific activities. Relatively high specific activities of radiolabeled proteins have been shown previously to result in a significant loss of immunoreactivity (7).

The H agglutination titers of antisera produced in this investigation were, overall, exceptionally high in comparison to those usually produced by intravenous immunization with highly motile *Salmonella* cells (4). Moreover, the H titers 2 months after the primary immunization were sufficiently high to make the antisera functional in homologous radioimmunoassays, and the titers of such antisera continued to rise thereafter. This is highly significant, as the performance of immunoassays is known to depend, above all, on the quality of the antisera used in such assays. It is of interest to note that in preliminary investigations, commercially available specific *Salmonella* H agglutinating antisera (Wellcome Diagnostics) were not functional in radioimmunoassays. This is presumably due to the different methodology used for antisera production and, consequently, much lower titers of specific antibodies in this commercial preparation.

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