JOURNAL OF CLINICAL MICROBIOLOGY, May 1981, p. 991-993
0095-1137/81/050991-03$02.00/0

Vol. 13, No. 5

New Method That Uses Binding of Immunoglobulin A to Group A Streptococcal Immunoglobulin A Fc Receptors for Demonstration of Microbial Immunoglobulin A Protease Activity

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Received 8 December 1980/Accepted 17 February 1981

A new method is described for the detection of bacterial immunoglobulin A (IgA) protease which splits IgA into Fab and Fc fragments. The method takes advantage of a recent finding that receptors for IgA Fc fragments occur commonly among type 4 group A streptococci. The bacterial preparation to be tested for protease activity was first incubated with radiolabeled purified IgA1 myeloma protein, and the proportion of radioactivity bound to a standard suspension of the streptococci was then measured. Since isolated Fab fragments do not bind to streptococcal IgA receptors, a decrease in the amount of radioactivity bound to the streptococci, as compared with the amount before digestion, indicates the presence of protease in the test preparation. Using this method, protease activity was detected in *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Streptococcus sanguis*, but not in *Escherichia coli* or *Branhamella catarrhalis*.

Immunoglobulin A (IgA) protease, capable of splitting IgA into Fab and Fc fragments, has been demonstrated in several bacterial species, including *Neisseria gonorrhoeae* (3, 9, 11), *Neisseria meningitidis* (3, 9, 11), *Streptococcus sanguis* (3, 9, 10), *Streptococcus pneumoniae* (3–5, 7), and *Haemophilus influenzae* (3–5, 7). All of the bacterial IgA proteases have proved to be specific for human IgA1; in other words, IgA2 and IgA of other species are not split (9, 12). The proteases of various bacterial species display some variability with respect to cleavage site, even though the site is always localized within the hinge of the IgA molecule (9). The IgA protease activity has hitherto been detected by incubation with IgA myeloma protein and demonstration of IgA cleavage by immunoelectrophoresis (9). This method is limited by the disadvantages of immunoelectrophoresis, e.g., insensitivity and lack of quantitation possibilities.

Receptors for IgA Fc fragments are common among type 4 group A streptococci (2, 13). In the present work, we took advantage of this interaction to detect microbial IgA proteases; the bacterial preparation to be tested was first incubated with radiolabeled IgA myeloma protein, and the proportion of radioactivity bound to a standard suspension of the streptococci was then measured. Since isolated Fab fragments do not bind to streptococcal IgA receptors (13), a decrease in the amount of radioactivity bound to the streptococci, as compared with the binding before digestion, indicates the presence of IgA protease in the test preparation.

Human IgA myeloma protein, purified by a combination of ammonium sulfate precipitation, ion-exchange chromatography, and preparative agarose gel electrophoresis, was kindly supplied by A. Grubb, Malmö General Hospital, Malmö, Sweden. Radiolabeling with ^{125}I was performed by the chloramine-T method (6).

Group A streptococci, type M4 (no. T4/596) were incubated overnight at 37°C in 100 ml of Todd-Hewitt broth. The bacteria were heat killed in a water bath at 56°C for 30 min, washed twice in phosphate-buffered saline (0.03 M phosphate, 0.12 M NaCl, pH 7.2), and suspended in 10 ml of phosphate-buffered saline. This "standard suspension" roughly corresponded to a concentration of 2.5×10^{10} bacteria per ml (1); as an excess of bacteria was used throughout the experiment (13), an exact quantitation was found not to be necessary. Preparations of *Branhamella catarrhalis*, *S. pneumoniae*, *Escherichia coli*, *N. meningitidis*, and *S. sanguis* (one strain of each) were obtained by culturing the bacteria overnight at 37°C in 10 ml of Todd-Hewitt broth. After centrifugation at 3,000 × *g* for 15

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min, the pellet, which was washed once in 10 ml of phosphate-buffered saline, and the supernatant were used in cleavage experiments. *H. influenzae* and *N. gonorrhoeae* were each cultured on six haematin agar plates at 37°C in 5% CO_2 atmosphere overnight. The gonococcal colonies were harvested with a sterile cotton swab and suspended directly in a solution of purified IgA1 myeloma protein. One of the plates inoculated with *H. influenzae* was harvested in a similar way, but the bacteria were suspended in 10 ml of Todd-Hewitt broth and incubated overnight at 37°C. The supernatant from this tube was saved for studies on protease activity. The five other plate cultures of *H. influenzae* were harvested, similarly incubated overnight in Todd-Hewitt broth, and washed in phosphate-buffered saline before use in cleavage experiments.

The bacterial pellets to be investigated for protease activity were suspended in 1.5 ml of radiolabeled IgA myeloma protein (10 μg), and the tubes were incubated at 37°C on a platform shaker for various periods. The suspension was then centrifuged at 3,000 × *g* for 15 min, and the supernatant (P) was saved for quantitation of binding of radioactivity to the type 4 group A streptococci. Proteases in supernatants from bacterial cultures (S) were detected by incubation of 100 μl of the preparation to be tested with 300 μl of ^{125}I-labeled IgA1 myeloma protein (8 μg) at 37°C on a platform shaker for various periods. S (100 μl) or P (300 μl) (equal to 2 μg of IgA1) was then mixed with 200 μl of a standard suspension of type 4 group A streptococci, the total radioactivity was counted, and the tubes were incubated at 22°C for 1 h. Phosphate-buffered saline (2 ml) containing 0.05% Tween 20 was added, the tube was centrifuged at 3,000 × *g* for 15 min, and the supernatant was aspirated off. The radioactivity in the pellet was then counted. Controls containing 0.15 M saline instead of the IgA protease preparation were included in each experiment. The percentage reduction in binding was calculated from the following formula: 100 × (radioactivity in control tube − radioactivity in test material tube)/(radioactivity in control tube).

A volume of 200 μl of the standard suspension of type 4 group A streptococci bound 65% of 2 μg of radiolabeled IgA myeloma protein added. Various bacterial preparations, i.e., supernatants and pellets from overnight centrifuged cultures of different species were incubated with IgA myeloma protein, after which the binding of radioactivity to 200 μl of the standard suspension of type 4 group A streptococci was assessed. After 4 h of incubation with gonococci the binding was reduced by 60%, and further incubation resulted in very little further reduction (Table 1). Assuming the molecular weights of IgA Fab and Fc fragments to be equal and the radioactive label of IgA to be evenly distributed, a complete digestion of IgA into Fab and Fc fragments would theoretically reduce the binding of radioactivity by 66%. Accordingly, the binding of IgA after exposure to gonococci would seem to imply that a complete digestion of IgA1 had occurred.

The digestion of IgA1 with *S. sanguis* and *S. pneumoniae* was maximal within 8 and 4 h, respectively. The finding of a maximum decrease in uptake by 51 and 54%, respectively, for these bacteria as compared with 66% for *N. gonorrhoeae* (Table 1) was of particular interest, as it has been demonstrated (3) that the IgA Fc fragments produced by protease(s) from *S. pneumoniae* and *S. sanguis* are larger than those produced by protease(s) from *N. gonorrhoeae*.

As also anticipated, bearing in mind other reports (7, 11), IgA protease activity was detected exclusively in the bacterial supernatants of *N. meningitidis* and *H. influenzae*, the maximum reduction levels being reached after 8 and 16 h, respectively (Table 1). The maximum reduction level achieved after 16 h with *H. influenzae* enzyme, 65%, was only slightly smaller than the theoretical value, 70.6%, as calculated from molecular weight determinations of these Fab and Fc fragments performed by others (3).

TABLE 1. *Detection of IgA protease activity in various bacterial preparations, using binding of IgA to type 4 group A streptococci*

Bacteria	Preparation incubated with IgA	% Reduction in binding of IgA to type 4 group A streptococci after:		
		4 h	8 h	16 h
N. gonorrhoeae	Pellet	60	62	66
N. meningitidis	Supernatant	42	54	54
	Pellet	0	0	0
H. influenzae	Supernatant	23	31	65
	Pellet	0	0	0
S. pneumoniae	Supernatant	54	54	54
	Pellet	54	54	54
S. sanguis	Supernatant	0	0	0
	Pellet	31	51	51
B. catarrhalis	Supernatant	0	0	0
	Pellet	0	0	0
E. coli	Supernatant	0	0	0
	Pellet	0	0	0

On the other hand, significant extracellular IgA protease activity was also found in *S. pneumoniae*, with a digestion rate identical with the intracellular activity. The absence of IgA1 protease activity in the strains of *B. catarrhalis* and *E. coli* was in agreement with results published by others (9).

In conclusion, the uptake of radiolabeled IgA1 on group A streptococci carrying IgA Fc receptors may be used for the detection and quantitation of IgA protease activity produced by extra- and intracellular bacterial enzymes. Since both subclasses of IgA are bound by streptococcal IgA Fc receptors (8), but IgA2 is not split by the specific IgA proteases, it is also possible to include radiolabeled IgA2 as a control for unspecific protease activity. The method would seem to offer a greater degree of sensitivity, compared with the immunoelectrophoretic methods hitherto used.

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