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Enzyme-Linked Immunosorbent Assay for Quantitating the Humoral Immune Response to the Colonization Factor Antigen of Enterotoxigenic *Escherichia coli*

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The enzyme-linked immunosorbent assay technique was used to quantitate, in milligrams per milliliter, anti-colonization factor antigen/I (CFA/I) immunoglobulin G (IgG) in acute- and convalescent-phase sera of individuals who experienced diarrhea associated with CFA/I-positive enterotoxigenic *Escherichia coli*. Purified CFA/I was used as antigen to coat polystyrene Microtiter plate wells for the determination of anti-CFA/I antibody. A reference anti-CFA/I IgG preparation was obtained by affinity chromatography of a high-titered serum with a CFA/I-Sepharose 4B column; IgG was the only class of immunoglobulin detectable in this serum as anti-CFA/I. Goat anti-human IgG conjugated to alkaline phosphatase was used in the enzyme-linked immunosorbent assay. Quantitation of IgG in the reference anti-CFA/I serum was achieved by comparison with a known sample of pure human IgG. Anti-CFA/I in test sera was quantitated by titration with CFA/I-coated Microtiter plate wells in the enzyme-linked immunosorbent assay, using a standard curve obtained with the reference anti-CFA/I serum. Anti-CFA/I IgG in paired sera was determined as percentage of total IgG by using the radial immunodiffusion technique to quantitate total IgG for each test serum. Diarrhea with isolation of CFA/I-positive enterotoxigenic *E. coli* was associated with a significant rise in serum anti-CFA/I IgG when these values were expressed as either milligrams of IgG per milliliter or as percentage of total IgG, although the response varied quantitatively and nonresponders were detected. None of the matched controls showed an anti-CFA/I IgG response. Further elucidation of the immune response to enterotoxigenic *E. coli* can now be accomplished by applying these methods to determine the class and specificity of immunoglobulins in external secretions such as saliva and intestinal contents.

Colonization factor antigens (CFA/I and CFA/II) are produced by specific serotypes of enterotoxigenic *Escherichia coli* (ETEC) (6, 9). These antigens are plasmid-mediated, surface-associated fimbrial structures which enable CFA-positive *E. coli* to adhere to the epithelial cell surface of the small intestine as a prerequisite to multiplication and establishment of an active infection (9-11). CFA/I and CFA/II are mannose-resistant hemagglutinins which are serologically distinct from each other (6) and from the common fimbriae (mannose-sensitive hemagglutinins) possessed by many strains of *E. coli* (8, 12). We have demonstrated humoral antibody responses to CFA/I (seroconversion) in individuals as the result of diarrheal illness with CFA/I-positive ETEC (10). Seroconversion was demonstrated with the conventional bacterial agglutination technique. However, because of the pathophysiology of ETEC disease (i.e., the noninvasive nature of ETEC), it is clear that antibody responses detectable in the external secretions and involving secretory immunoglobulins are probably more relevant to immunoprotection than serum antibody. During recent years the enzyme-linked immunosorbent assay (ELISA) has been used extensively to detect and quantitate immunoglobulins against bacterial antigens (1, 4, 13, 14). Furthermore, the ELISA procedure is adaptable to the quantitation of specific classes of immunoglobulins (14). For these reasons we have developed an ELISA procedure based upon the use of purified CFA/I antigen (7) for the quantitation of anti-CFA/I antibody in either serum or external secretions. This report describes seroconversion with respect to the CFA/I antigen as a function of milligrams of anti-CFA/I immunoglobulin G (IgG) per ml in serum.

MATERIALS AND METHODS

Preparation of CFA/I-Sepharose for affinity chromatography. Purified CFA/I antigen (7) was coupled to cyanogen bromide (CNBr)-activated Seph-

arose 4B (Pharmacia Fine Chemicals, Sweden) as follows. One gram of CNBr-Sepharose 4B was added to 10 ml of 1.0 mM HCl. The gel was washed with 200 ml of 1.0 mM HCl and then dried. A total of 28 mg of CFA/I antigen, as determined by Lowry protein analysis (7), was added to 10 ml of 0.1 M borate buffer plus 0.5 M NaCl (pH 8.3). The CNBr-Sepharose 4B was added to this solution and mixed, in an end-over-end shaker, for 1 h at room temperature. The gel was then dried under vacuum and washed with 200 ml of borate buffer. Unreacted active sites on the gel were blocked by mixing the gel with 1.0 M ethanolamine (pH 9.0) for 2 h at room temperature. Excess blocking reagent was removed by alternately washing the gel with 100 ml of 0.1 M acetate buffer plus 0.5 M NaCl (pH 4.0) and with 100 ml of the 0.1 M borate buffer. The washing procedure was repeated three times, and the gel was stored at 4°C in borate buffer.

Preparation of anti-CFA/I IgG. Immune serum against the purified CFA/I antigen was prepared by the subcutaneous injection of one of us (D.J.E.) with 100 μg of antigen. A booster dose was given subcutaneously 4 weeks after the first injection. Serum obtained 4 weeks after the second injection was shown to have a very high titer (1:1,024) against CFA/I-positive *E. coli* as judged by the bacterial agglutination tube test. One milliliter of this serum was added to 8.0 ml of borate buffer and mixed with the CFA/I-Sepharose gel, which had been washed in borate buffer. The mixture was shaken for 30 min at room temperature. The gel was then washed with 200 ml of borate buffer to remove serum components that had not reacted with the CFA/I antigen coupled to the gel. Anti-CFA/I antibody was eluted from the CFA/I-affinity gel with 6.0 M sodium thiocyanate, and the eluate was dialyzed against 0.1 M phosphate-buffered saline (PBS) for 48 h at 4°C with several buffer changes. Human serum without any demonstrable anti-CFA/I titer, as judged by bacterial agglutination, was used to determine the specificity of the CFA/I-Sepharose gel. After elution of the antibody the CFA/I-Sepharose gel could be stored at 4°C for 3 months and still be used to isolate anti-CFA/I antibody.

Quantitation of IgG in the eluate from CFA/I-Sepharose gel. The concentration of anti-CFA/I IgG in the eluate was determined by the amount of inhibition produced in an ELISA in comparison to the inhibition produced by known concentrations of IgG in the same assay. Equal volumes of doubling dilutions of a human IgG reference sample (Hyland Laboratories, Inc., Costa Mesa, Calif.) or of the CFA/I-affinity gel eluate were mixed in test tubes with equal volumes of goat anti-human IgG (gamma chain specific, Cappel Laboratories) conjugated to alkaline phosphatase (anti-IgG–enzyme conjugate); the final dilution of this conjugate was 1:500. These reaction mixtures were incubated at 37°C for 30 min. The wells of a polystyrene Microtiter plate (Flow Laboratories, Rockville, Md.) were coated with human IgG (20 μg of the IgG reference sample per 200 μl of buffer per well) and incubated for 18 h at 37°C. After the coated Microtiter wells had been washed three times with 0.1 M PBS plus 0.05% Tween 20 (PBS-Tween), 200 μl of the IgG–anti-IgG–enzyme conjugate mixtures (above reaction mixtures) was added. The plates were kept at room temperature for 2 h, the wells were washed with PBS-Tween, and 200 μl of the enzyme substrate (*para*-nitrophenyl phosphate at 1.0 mg/ml) was added. The reaction was allowed to proceed for 100 min at 37°C before being stopped by the addition of 50 μl of 3 M NaOH. Alkaline phosphatase product was determined by measuring the absorbance, at 400 nm, of each reaction mixture by using a Beckman double-beam spectrophotometer. The value representing zero inhibition was produced with enzyme conjugate to which no IgG had been added. Microtiter wells not coated with IgG were used to demonstrate that nonspecific adsorption of IgG, anti-IgG–enzyme conjugate, or enzyme conjugate alone did not occur.

ELISA procedure for quantitating anti-CFA/I IgG. Microtiter plate wells were coated with 200 μl of purified CFA/I antigen (1.0 μg/ml) by incubating the plates at 37°C for 18 h. The plates were washed with PBS-Tween before the addition of 200 μl of the test serum samples diluted in PBS-Tween, after which the plates were allowed to stand for 2 h at room temperature. After a further washing with PBS-Tween, 200 μl of the anti-IgG–enzyme conjugate was added to the wells, and the reaction was allowed to proceed for 2 h at room temperature. After a final washing with PBS-Tween, 200 μl of enzyme substrate was added to the wells, and the plates were incubated at 37°C for 100 min. Finally, the reaction was stopped by the addition of 50 μl of 3.0 M NaOH, and the absorbance at 400 nm was determined for each reaction mixture. Each test was performed in duplicate and also included known positive and negative anti-CFA/I sera. Wells coated with no antigen were used to ensure that nonspecific adsorption of reagents did not occur.

Quantitation of total amount of IgG in test samples of serum. The radial immunodiffusion technique (Hyland Laboratories) was used to quantitate the total amount of IgG in each serum sample assayed for specific IgG by the ELISA. The Hyland IgG reference sample served as the standard for these determinations.

Stool and serum samples. One group of serum samples was derived from adults with diarrhea in which CFA/I-positive ETEC was isolated. Another, control, group of sera was derived from age-matched healthy individuals with stool specimens negative for CFA/I-positive ETEC. Preimmune serum samples were collected, when possible, from the participants before onset of disease. Acute- and convalescent-phase serum samples were obtained from individuals when they developed diarrhea and from 1 to 4 weeks later, respectively. For those individuals who did not become ill, blood samples were taken at the time ETEC was found in the stools and from 1 to 4 weeks later. All sera were stored at −65°C.

Statistical analysis. Statistical analysis of the results was determined by the Student *t* test; values < 0.05 were considered significant.

RESULTS

Optimal concentration of CFA/I antigen for quantitating anti-CFA/I by the ELISA.

The direct ELISA method was used to determine the optimal concentration of CFA/I antigen to coat Microtiter plate wells for reaction with IgG anti-CFA/I. It was found that, within the range of concentrations of CFA/I from 10 to 0.01 μg/ml, the optimal concentration required to coat the Microtiter wells was 1.0 μg/ml (Fig. 1). Concentrations greater than 1.0 μg/ml did not result in an increase in absorbance at 400 nm; at concentrations lower than 1.0 μg/ml a decrease in absorbance did occur.

Adsorption of the antigen to the Microtiter plate wells occurred best at 37°C after 18 h. Incubation of the plates for 48 h at 37°C or 18 h at 4°C resulted in decreased adsorption of the antigen (Table 1).

Optimum dilution of goat anti-human IgG-enzyme conjugate. All test samples were assayed, using the goat anti-human IgG conjugate at a final dilution of 1:900. At higher dilutions a significant decrease in absorption at 400 nm was observed, whereas at lower dilutions no increase in the sensitivity of the assay was found (Fig. 2).

Affinity chromatography of anti-CFA/I IgG. A total of 28 mg of CFA/I antigen was added to the CNBr-Sepharose gel. After incubation it was found that approximately 25 mg (89%) of the antigen had been coupled to the gel. This protein was not removed by a repeated washing of the gel with borate buffer (pH 8.0) and acetate buffer (pH 4.0).

Human immune serum, obtained by immunization with purified CFA/I antigen, was mixed with the CFA/I-Sepharose gel beads; this serum had previously been shown by the ELISA to contain 1.512 mg of IgG directed against CFA/I (see below) per ml. Excess, unreacted antibody was removed by filtration under vacuum, and this filtrate contained 0.908 mg of specific IgG per ml. From this it was calculated that the antigen-coupled gel retained 40% of the anti-CFA/I immunoglobulin present in the immune serum. Anti-CFA/I immunoglobulin could be eluted from the CFA/I-affinity gel using 6.0 M sodium thiocyanate, whereas no immunoglobulin could be eluted from the gel after passage of a serum which was negative for anti-CFA/I activity when tested by the ELISA and by the bacterial agglutination method. The concentration of IgG in the anti-CFA/I column eluate was determined by comparison of the inhibition produced by this sample with that produced by a reference IgG sample, when these were mixed with anti-IgG-enzyme conjugate that was subsequently used to detect IgG in an ELISA. No inhibition was observed when this IgG-coated plate was incubated with the enzyme conjugate alone, resulting in an optical density at 400 nm of 1.07 (see Fig. 3). Inhibition produced by the highest concentration of the reference IgG sample resulted in an optical density of 0.10 at 400 nm. Therefore, an absorbance of 0.48 at 400 nm represents 50% inhibition, at which point the

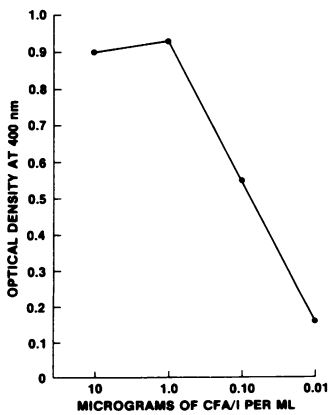

FIG. 1. *Determination of the optimum concentration of CFA/I antigen for coating Microtiter plate wells to perform the ELISA. Coating was at 37°C for 18 h.*

TABLE 1. *Effect of incubation time and temperature on the coating of Microtiter plate wells with CFA/I antigen*

Time of incubation (h)	Temp of incubation (°C)	Optical density at 400 nm[a]
18	37	0.93
18	4	0.45
48	37	0.88

[a] ELISA results obtained with 1.0 μg of CFA/I per ml, using an anti-CFA/I serum at a dilution of 1:800.

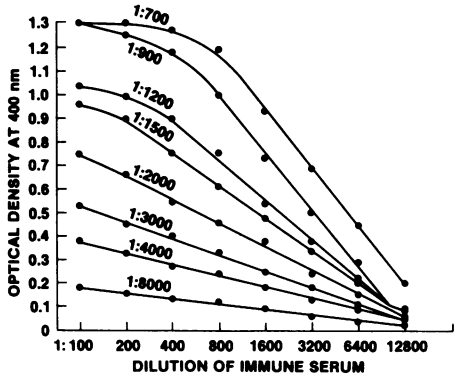

FIG. 2. *Determination of the optimum dilution of goat anti-human IgG-alkaline phosphatase conjugate for titrating human anti-CFA/I IgG in sera. Each curve represents a different dilution of the conjugate.*

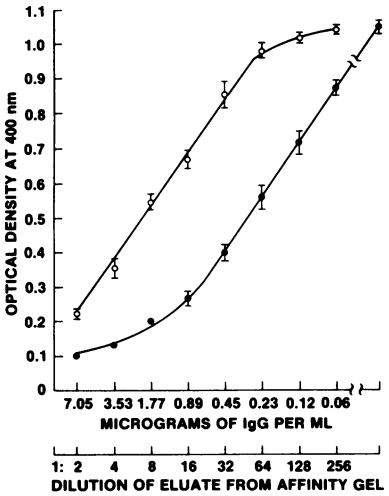

FIG. 3. *Quantitation of IgG (in micrograms per milliliter) in a purified anti-CFA/I sample by inhibition of the reaction between anti-IgG–enzyme conjugate and IgG bound to Microtiter plate wells. Doubling dilutions of the anti-CFA/I serum (column eluate) were preincubated with a constant amount of the conjugate, and samples were transferred to IgG-coated wells for performance of the ELISA. The results are represented by the upper curve* (○). *Conjugate was also preincubated with known concentrations of human IgG before the ELISA; these results are represented by the lower curve* (●). *The highest point on the lower curve shows the result obtained when the conjugate was preincubated without IgG; this point represents zero inhibition in the test. The bars show the range of optical density values obtained for each test sample.*

concentration of IgG in the reference sample was 0.39 μg/ml. The anti-CFA/I IgG column eluate sample produced 50% inhibition at a final dilution of 1:7, and therefore the concentration of IgG in this eluate was calculated to be 2.8 μg/ml (Fig. 3). This value is small, due to the inefficiency of the sodium thiocyanate elution procedure, but adequate (see Fig. 4).

Calibration of a standard curve for quantitating anti-CFA/I in human serum. Doubling dilutions of the purified anti-CFA/I IgG were employed in an ELISA to produce a standard curve, using 1.0 μg of CFA/I per ml to coat the Microtiter wells and a 1:900 dilution of the conjugate. The resultant curve was linear between the values of 2.80 and 0.087 μg of IgG per ml (Fig. 4). The standard curve was calibrated on two separate occasions and found to give identical values. Also, no absorption was observed with enzyme conjugated to goat anti-human IgM or to goat anti-human IgA, indicating that the eluate from the affinity gel contained anti-CFA/I belonging only to the IgG class of antibody.

Quantitation of anti-CFA/I IgG in serum samples. Serum samples from 28 individuals were examined by the indirect ELISA for anti-CFA/I antibody. Figure 5 shows the type of results obtained by plotting the ELISA results (absorbance at 400 nm) against serum dilution. In all cases the curves exhibited a linear relationship between an absorbance of 0.10 and 0.90, and the linear portions of the curves from each individual were parallel. The concentration of anti-CFA/I IgG in each sample was calculated by determining the optical density, at 400 nm,

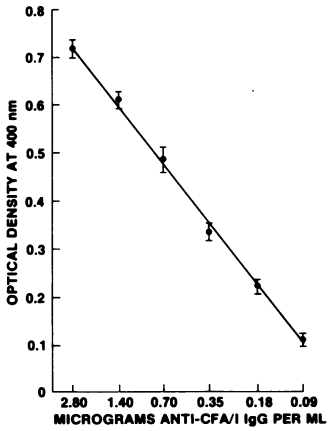

FIG. 4. *Standard curve showing the linear relationship between anti-CFA/I IgG concentration (micrograms per milliliter) and optical density at 400 nm produced with CFA/I-coated Microtiter plate wells and anti-human IgG–enzyme conjugate (1:900 dilution). The bars show the range of values obtained for each test sample in the ELISA.*

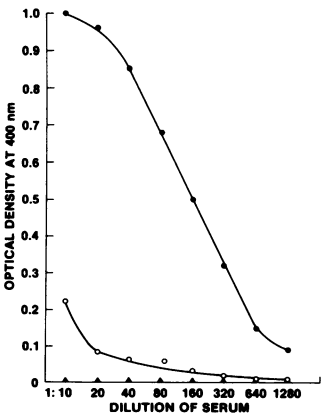

FIG. 5. *Determination of anti-CFA/I IgG in acute-phase* (○) *and convalescent-phase* (●) *sera by the indirect ELISA. Values representing the linear portion of the curve (usually the dilution at the midpoint) were used to calculate the amount of anti-CFA/I IgG by reference to the standard curve shown in Fig. 4.*

at the midpoint of each curve and by reference to the standard curve shown in Fig. 4. A plateau was often seen at the higher concentrations of IgG. Repeated tests with Microtiter wells coated with antigen on different days showed that the values for anti-CFA/I IgG concentration of any serum sample did not exceed 10%.

Tables 2 and 3 show the level of anti-CFA/I IgG in each of the serum samples observed and, also, this value expressed as a percentage of total IgG in the respective sera. For those individuals from whom CFA/I-positive ETEC were isolated during illness, the range of anti-CFA/I IgG in the acute-phase serum samples was from below 0.002 to 0.131 mg/ml, with a mean value of 0.024 mg/ml for those with detectable anti-CFA/I IgG; two persons had no detectable anti-CFA/I IgG antibody. In the convalescent-phase serum samples, anti-CFA/I IgG ranged from 0.003 to 0.840 mg/ml, with a mean value of 0.259 mg/ml. Analysis of these results suggested that the rise in the quantity of anti-CFA/I antibody for the paired sera was significant at the level of $P < 0.0035$. When these values are expressed as percentage of total IgG (Table 2), the range in the acute-phase sera is 0.01 to 0.94%, with a mean value of 0.17%, and for the convalescent-phase sera the range is 0.02 to 9.91%, with a mean value of 2.27%. Thus the increase in anti-CFA/I expressed as percentage of total IgG (acute- versus convalescent-phase sera) is significant at the level of $P < 0.01$.

Table 3 shows the levels of anti-CFA/I IgG in the group of individuals without diarrheal disease. For the "acute"-phase serum samples, the quantity of specific antibody in the serum ranged from 0.002 to 0.016 mg/ml, with a mean value of 0.006 mg/ml. The "convalescent"-phase sera exhibited a range of anti-CFA/I IgG from 0.002 to 0.019 mg/ml; the mean value was 0.006 mg/ml. In terms of the proportion of specific IgG to total IgG in these samples, the range for the "acute" samples was 0.02 to 0.22%, with a mean of 0.08%, and for the "convalescent" samples the range was 0.02 to 0.27%, with a mean of 0.09%. Statistical analysis of these results revealed no significant difference between the values determined for the "acute"- and "convalescent"-phase sera obtained from the control (non-ill) group.

TABLE 3. *Anti-CFA/I IgG, total IgG, and percentage of IgG that is anti-CFA/I in acute- and convalescent-phase (time- and age-matched control) sera of individuals who did not experience diarrhea and who were negative for CFA/I-positive ETEC*

Subject no.	Acute-phase serum			Convalescent-phase serum		
	Anti-CFA/I IgG (mg/ml)	Total IgG (mg/ml)	% Anti-CFA/I[a]	Anti-CFA/I IgG (mg/ml)	Total IgG (mg/ml)	% Anti-CFA/I
1	0.005	9.0	0.06	0.004	8.0	0.06
3	0.006	8.8	0.07	0.004	9.7	0.05
8	0.003	7.8	0.04	0.003	8.3	0.04
9	0.002	12.8	0.02	0.002	12.8	0.02
12	0.000	9.1	0.00	0.000	7.1	0.00
13	0.000	6.8	0.00	0.000	7.0	0.00
14	0.016	7.4	0.22	0.019	7.3	0.27
18	0.000	8.4	0.00	0.000	8.0	0.00
20	0.000	10.4	0.00	0.000	9.8	0.00

[a] Percentage of anti-CFA/I = CFA/I-specific IgG divided by total IgG × 100.

TABLE 2. *Anti-CFA/I IgG, total IgG, and percentage of IgG that is anti-CFA/I in acute- and convalescent-phase sera of individuals with diarrhea associated with CFA/I-positive ETEC*

Subject no.	Acute-phase serum			Convalescent-phase serum		
	Anti-CFA/I IgG (mg/ml)	Total IgG (mg/ml)	% Anti-CFA/I[a]	Anti-CFA/I IgG (mg/ml)	Total IgG (mg/ml)	% Anti-CFA/I[a]
2	0.005	7.5	0.07	0.704	7.4	9.91
4	0.005	14.8	0.04	0.336	13.6	2.47
5	0.131	13.8	0.94	0.236	22.2	1.06
6	0.094	17.4	0.54	0.236	17.4	1.35
7	0.003	8.4	0.04	0.022	8.3	0.27
10	0.003	28.0	0.01	0.003	15.6	0.02
11	0.000	10.4	0.00	0.000	17.4	0.00
15	0.046	15.2	0.30	0.840	17.8	4.70
16	<0.002	6.8	<0.03	0.145	6.8	2.13
17	0.002	11.2	0.02	0.350	10.2	3.40
19	0.000	9.8	0.00	0.128	9.8	1.30
21	0.004	13.8	0.03	0.342	13.8	2.50
22	0.002	10.0	0.02	0.003	10.0	0.03
23	0.007	10.4	0.07	0.031	21.2	0.14
25	0.004	7.0	0.06	0.252	9.8	2.57

[a] Percentage of anti-CFA/I = CFA/I-specific IgG divided by total IgG × 100.

DISCUSSION

The ELISA, first described by Engvall and Pearlmann (5), provides a sensitive method for determining the presence of specific classes of antibody directed against specific bacterial antigens (3, 4, 14). The availability of purified CFA/I antigen (7) has enabled us to use the ELISA to quantitate the levels of anti-CFA/I IgG antibody in the serum of individuals exposed to ETEC. To use the ELISA to detect anti-CFA/I antibody, it was necessary first to determine optimal conditions for performing the assay. It was found that purified CFA/I antigen adsorbed optimally to polystyrene Microtiter plates at a concentration of 1.0 μg/ml with incubation at 37°C for 18 h. The decrease in reactivity observed with higher coating concentrations has been previously observed (14) and is thought to be due to elution of the antigen from the plate during the first antigen-antibody reaction used in the test.

CFA/I-specific IgG was detected using goat anti-human IgG, conjugated to alkaline phosphatase, at a final dilution of 1:900. The working dilution of each batch of enzyme conjugate must be determined before use, since different batches possess different optimal working concentrations. Using the technique that we describe here to calculate the concentration of anti-CFA/I IgG in terms of milligrams per milliliter, it was unnecessary to use a single batch of enzyme conjugate for all test samples because a simple correction factor (obtained by using a standard anti-CFA/I IgG sample) could be determined for each batch of conjugate.

Quantitation of anti-CFA/I IgG in test sera in terms of milligrams per milliliter did necessitate procurement of a sample of purified anti-CFA/I IgG for construction of a standard curve. This was accomplished by using purified CFA/I antigen as a ligand, coupled to CNBr-activated Sepharose 4B, to purify the anti-CFA/I antibody from a sample of human hyperimmune serum. The quantity of IgG in this anti-CFA/I preparation was determined by inhibition, in an ELISA, of an IgG–anti-IgG reaction in comparison with a reference curve determined with known quantities of IgG obtained as purified material from a commercial source. The standard curve produced by the purified anti-CFA/I preparation showed good reproducibility and measured concentrations of specific IgG down to 2.80 μg/ml. Also, tests performed by the indirect ELISA procedure with goat anti-human IgM or goat anti-human IgA as the enzyme conjugate showed that all of the detectable anti-CFA/I antibody in the hyperimmune serum was IgG.

In general, persons who had diarrhea known to be associated with ETEC showed a significant rise in anti-CFA/I IgG in their sera as measured in milligrams of specific antibody per milliliter. There was, however, variation in the response of different individuals; one person showed no detectable levels of anti-CFA/I IgG in either the acute- or the convalescent-phase serum. On the other hand, no significant rises in anti-CFA/I IgG levels were observed in individuals who showed no signs of clinical illness and from whom CFA/I-positive ETEC could not be isolated.

Although the number of individuals participating in this study was small, the rise in serum anti-CFA/I IgG was significant for the group in which CFA/I-positive ETEC was isolated; this suggests that this antibody response is a good indicator of the agent responsible for the diarrhea in these individuals. The role of serum IgG in protection or recovery from ETEC diarrheal disease is questionable because there is no tissue invasion by the CFA/I-positive ETEC, suggesting that immunity or recovery from ETEC diarrhea is likely to be mediated by secretory antibodies such as secretory IgA. However, the presence of anti-CFA/I IgG in serum appears to be a good indicator of an immune response and thus might result from "leakage" of the antigen into the peripheral lymphoid system during that time in which the secretory immunoglobulin system is being stimulated.

In summary, we have described here a method for quantitating the levels of antigen-specific IgG in serum by using the ELISA procedure; the resultant values can be expressed in terms of milligrams per milliliter of serum or as the proportion of specific IgG to total IgG in the serum after determining the total concentration of serum IgG in each test sample. The quantitation of secretory IgA in external secretions such as urine, milk, and saliva by the ELISA has been described by other investigators (1, 2). We plan to apply this procedure to quantitate the concentrations of antigen-specific immunoglobulins (for example, anti-CFA/I, anti-CFA/II, and anti-enterotoxin) in external secretions of individuals exposed to these ETEC antigens as a consequence of natural or artificially induced ETEC diarrhea. Hopefully, this will facilitate a determination of (i) the class of immunoglobulin most important to immunity against ETEC diarrhea, and (ii) the relative role of ETEC antigens (for example, enterotoxin versus colonization factor antigens) in immunoprotection.

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