Elimination of Interfering Activity in Serum Samples in the Chinese Hamster Ovary Pertussis Serology Assay

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An interfering substance in various frozen serum samples was observed to inhibit the adhesion of Chinese hamster ovary (CHO) cells to microplate surfaces during a CHO pertussis neutralization test, resulting in wells that lacked cells or wells with dead cells after 2 days of incubation. The interfering activity in the serum could be eliminated by (i) transferring cells to other wells after their initial incubation, (ii) adding fetal calf serum (FCS) to the sample dilution buffer, (iii) preincubating microplates with FCS, or (iv) preincubating the samples at 4°C for 5 days. Preincubating the samples at 4°C for 5 days reduced the interfering activity in only some of the samples. Adding serum to the sample dilution buffer or preincubating the microplates with serum did not influence the antibody titers in the serum samples. The method described may be used for routine applications.

Toxin from Bordetella pertussis has been observed to induce clustering of Chinese hamster ovary (CHO) cells (4). The clustering effect induced by pertussis toxin (PT) can be used when samples are analyzed in clinical trials of vaccines containing pertussis toxoid, since neutralizing antibodies against pertussis toxoid have been observed to inhibit this effect (1).

Cells are initially seeded into wells of titer plates, and subsequently, various concentrations of serum samples that have been preincubated with pertussis toxoid are added to the adherent cells in the wells, according to the method described by Hewlett et al. (4). This method works well with a few samples, but with multiple samples to be analyzed, like those in clinical trials, it is convenient to titrate serum samples with pertussis toxin in the titer wells and subsequently add cells to the mixture in the wells (1). This sequence of additions to titer wells is the same as that used in the methods to determine protection against diphtheria toxin and poliovirus, where serum samples are titrated with diphtheria toxin or poliovirus in titer wells before the addition of Vero cells (5, 6).

When serum samples are analyzed for neutralizing antibodies to pertussis toxoid by the method where cells are added to preincubated mixtures of serum samples and pertussis toxoid, we have observed that interfering activity in quantities ranging from a few percent to 50% of the samples interferes with reading of the cells. This is in agreement with observations by others who have observed toxic activity in serum samples (1–3, 7, 8). The interfering activity could be abolished by using the original method described by Hewlett, but since this method is too laborious to use in clinical trials, we have, instead, improved the method where cells are added to the wells subsequent to the serum samples and the pertussis toxoid.

MATERIALS AND METHODS

Cells and media. CHO cells purchased from ATCC (CHO-K1, ATCC CCL-61) were cultured in a culture medium consisting of Ham’s F12K (Gibco) supplemented with 8% heat-inactivated fetal calf serum (FCS; Gibco), 0.001% gentamicin, and 0.15% sodium bicarbonate in 175-cm² flasks at 37°C in an atmosphere containing 4 to 6% CO₂ (7). The cells were subcultured twice a week. A single-cell suspension was obtained from confluent monolayers of CHO cells by treatment with a solution of 0.01% trypsin and 0.02% versen in phosphate-buffered saline (pH 7.4).

Dilution medium consisting of Ham’s F-12K with 2% FCS was used to dilute serum samples and pertussis toxin (7).

Pertussis toxin. The B. pertussis toxin was a gift from Baxter (Deerfield, IL). It was calibrated against an international reference preparation (catalog no. 90/518; NIBSC, Co.).

Serum samples. The serum samples were from a clinical trial (unpublished data) where the efficacies of two vaccines containing diphtheria, tetanus, and pertussis toxoids and inactivated poliovirus vaccines were compared when they were administered simultaneously with Haemophilus influenzae type b conjugate vaccine (polyribosylribitol phosphate conjugated to tetanus toxoid). One of the vaccines contained inactivated poliovirus vaccine produced from Cercopithecus monkey kidney cells, and the other vaccine contained inactivated poliovirus vaccine produced from Vero cells cultured in the absence of FCS. The vaccination schedule was 2, 3.5, 5, and 16 months, and serum samples were obtained at 6, 16 and 17 months.

Pertussis NT. The pertussis neutralization test (NT) used here followed the principles described previously (1, 2) but included the modifications described below. The method is based on the observation that PT induces clustering of CHO cells, which is inhibited by antibodies. Briefly, twofold dilutions, from a dilution of 1:2 to a dilution of 1:4,096 (50 μl/well, duplicate determination), of serum samples were made in dilution medium. Fifty microliters of dilution medium containing 0.25 μg of PT, equal to fourfold the minimum amount that induces clustering activity, was added to each well, and the mixture was incubated for 3 h at 37°C. Subsequently, 100 μl of CHO cells in culture medium (1 × 10⁶ cells/ml) was added to each well (and exposed to interfering activity/toxicity” in the wells) and incubated for 2 h at 37°C. The medium was replaced by culture medium (200 μl/well), and the 96-well culture microplates (Nunc) were sealed with tape and incubated at 37°C for 48 h (± 6 h). In some tests, the Nunc microplates were replaced with fibronecin-coated microplates from BD Biosciences (BD BioCoat Fibronectin Cellware; cat. no. 354409), or plates were precoated with 5% human serum albumin (Statens Serum Institut).

Controls. A serum pool with a high titer and a serum pool with a low titer were used as titer controls. CHO cells without toxin served as negative control.

Fixation. The cells were incubated with 50 μl/well fixation liquid (10% glacial acetic acid-90% ethanol) for 2 min. The liquid was removed, and the microplates were air dried for 15 min. Fifty microliters of undiluted Giemsa-azure-cosin-methylene blue stain solution (Merck) was added, followed by 5 min of incuba-
The microplates were washed three times with water and dried under a hood for at least 1 day. The microplates were then covered with tape and photographed at a magnification of 100, using an Olympus CK-2 microscope equipped with a Nikon DS camera.

Evaluation of plates. Titers were expressed as the reciprocal of the highest serum dilution resulting in 100% neutralization of the clustering effect of the toxin. The result is given as the mean of the duplicates.

Statistical analysis of sample titers obtained by the Gillenius method (1) versus that obtained by the improved method was performed with GraphPad Prism 3 software.

RESULTS

When a total of 550 frozen serum samples were analyzed in the pertussis clustering assay described above, 152 samples could not be read due to interfering (“toxic”) activity in the samples, as described by others (4). The interfering activity in these samples appeared from the third (1:8) to the sixth (1:64) dilution but was infrequently observed in the first and second dilutions (1:2 and 1:4, respectively). Since the interfering activity remained in the culture wells after the medium was changed to culture medium, the effect was thought to be caused by an insoluble substance adhering to the wells. In the initial experiments, some samples with the interfering activity lost their ability to interfere when they were stored for more than 5 days at 4°C between experiments. This effect was reproducible for some samples, but since not all samples lost the interfering activity by incubation at 4°C, we were forced to improve the method by other means.

When we resuspended the culture medium vigorously in the wells at the time the medium was changed, we observed that cells in some of the wells were loosened and could be transferred to other wells (Fig. 1). When the cells were inspected after 48 h, no effect from the serum samples was observed for the transferred cells. The interfering effect on the serum samples probably originated from an inhibition of the cells to adhere to the plastic surface of the wells, since only cells from wells incubated with dilutions of serum known to interfere with the reading of the cells (dilutions 1:8 to 1:64) could be transferred semiquantitatively from one well to another. By using a
combined reading of the rows where the cells were originally seeded and the rows to which the cells were transferred, we were able to determine the pertussis antibody titers even with the interfering activity in the serum samples (Fig. 1). Neutralization of toxin is observed at a 1:16 dilution of serum (the titer equal to 16 in the serum sample); a minor amount of clustering activity is observed at a 1:32 dilution, and at a 1:64 dilution, half-maximal clustering has been observed. No inhibition of clustering activity is observed at a serum dilution of 1:128. However, since this method is time consuming and the cells could not be transferred quantitatively, we wished to improve the method further.

The observation that most of the samples from sera that otherwise had interfering activity were without interfering activity in the initial dilution suggested that high concentrations of serum could inhibit the interfering effects in the serum samples. Therefore, a dilution series from interfering serum samples was made in dilution buffers with increasing amounts of FCS (2%, 4%, 8%, 16%, 32%, and 64%), and the assay was performed as described previously. Interfering activity was observed when dilution buffers with 2%, 4%, and 8% FCS were used to dilute the serum samples; with 16% FCS, the interfering activity was almost eliminated, and with 32% and 64% FCS, we observed no interfering activity in the serum samples (results not shown). Since the dilution buffer with 32% FCS extensively stimulated the growth of the CHO cells, making it difficult to obtain a cell density that could be easily read after 2 days of culture, the method was modified further.

We found that by coating the wells to be used in the assay with 100 µl of Ham’s F-12K supplemented with 40% FCS at 4°C overnight, all interfering activity in the samples was inhibited. Figure 2 shows a titration of the sample that was titrated in Fig. 1; for all dilutions, the cells showed high adherence (a flattened morphology with easily distinguished cytoplasm), indicating that the coating of 40% FCS eliminated the interfering activity of the sample serum, and a titer of 32 was read, which is close to the titer shown in Fig. 1. The small difference in titer may be explained by a day-to-day variation, since a small variation in titer may be observed when samples are retested by the same method. When the 152 samples showing interfering activity (out of 550 samples) were retested with the improved method, all samples could be read without problems.

To corroborate that the improvement in methodology did not impact the results obtained, titers for 54 samples with noninterfering activity, analyzed by the original method, were compared with titers of the same samples analyzed by the improved method (Fig. 3). No differences in titers were observed (slope of regression line, 1.043; with 95% confidence limits, 0.9443 and 1.141).

Since fibronectin and albumin are constituents in FCS, we supposed that the presence of these proteins might cause increased adhesion and subsequent elimination of interfering activity. The hypothesis was tested using fibronectin- or albumin-coated wells. No effect of the interfering activity was observed when albumin was used, but the interfering activity was abolished when microplates coated with fibronectin were used. However, when serum samples with the interfering activity were titrated on these microplates, no clustering effect from pertussis toxin was observed when the titration endpoint was reached. Therefore, these trays could not be used as a substi-
In summary, the modified method is as follows. (i) Titer wells are precoated overnight by incubation at 4°C with 100 μl of a dilution of 40% FCS in Ham’s F-12K medium. (ii) Wells are emptied and filled with 50 μl of dilution medium containing 2% FCS. Fifty microliter quantities of samples, each in duplicate, are added to the left column of the culture microplates, and twofold dilution series are prepared across the plates. (iii) Fifty microliters of PT (approximately fourfold the minimum amount that induces clustering activity, 5 ng/ml) is added into the culture medium (culture medium consists of 0.15% sodium bicarbonate, 0.001% gentamicin, and 8% FCS in Ham’s F-12K medium) and incubated at 37°C. (iv) One-hundred microliters of CHO cells (1 × 10^5 cells/ml) is added to the sample dilutions and incubated for 3 h at 37°C. (v) After 2 h, medium is replaced with 200 μl of culture medium, and 48 h later, cells are stained as described in Materials and Methods.

**DISCUSSION**

In the literature, various frequencies of toxicity in serum samples have been described, ranging from “a few toxic samples” (1–3) to 30% of the samples being toxic (7). Commonly, the toxic effect seems to be found in human sera that have been repeatedly handled and refrozen. The variation observed by others seems to correlate with the amount of FCS present in the dilution medium, since the amount of FCS in the dilution medium has been either 2% FCS (7) or 10% FCS (1–3). In addition, one report has described the replacement of Ham’s F-12 medium by medium 199 in order to reduce toxicity of the serum samples (3). Since the toxic activity observed by others is the same interfering activity that we have observed, we hypothesize that the two activities are alike.

In our assay, we encountered problems concerning the interfering activity in the serum samples when they were analyzed in the CHO microplate pertussis serology assay, and we found that the interfering activity in the samples seemed to increase after they were frozen. We observed that the storage of some thawed samples at 4°C for 5 days reduced the interfering activity in serum samples. Since the interfering activity was not reduced in all samples with interfering activity, and occasionally, up to 50% of the samples had interfering activity, we were forced to improve the CHO-cell serology assay.

We observed the following. (i) The interfering effect in serum samples was due to inhibition of the adherence of CHO cells to the well surfaces, since cells in wells with interfering activity samples could be easily transferred to other wells where they could adhere. The interfering effect was lost after the cells were transferred. (ii) An increased amount of FCS in the dilution medium reduced the interfering activity in the serum samples, but FCS had to be present at a concentration of at least 32% for the interfering effects to be avoided completely. To avoid the side effect in which high amounts of serum also stimulated cell growth, we improved the method by coating the wells with 40% FCS. This modification had no effect on cell growth or determination of pertussis antibody titer.

Initially, the interfering activity could be assumed to be an activation of the apoptotic cascade, but since the effect, unlike the apoptotic “trigger,” is reversible (when cells are transferred to other wells, the activity is lost), this assumption seems to be unlikely. The reason that the inhibition of adherence leads to interfering activity in the CHO cells could be the lack of activation of the α5β1 fibronectin receptor on the CHO cells, since activation of this receptor is known to be of importance for the survival of CHO cells (9).

It could be hypothesized that the interfering activity was due to proteolytic activity in the toxic serum samples, but this suggestion seems unlikely, since the activity was not observed at high concentrations of serum samples, and was inhibited by the addition of serum, could be that the interfering activity competes with a serum constituent for binding to the surface of the plates. Inhibition of the binding of CHO cells to the plates might be dependent on the absolute as well as the relative concentrations of serum components and the interfering activities. Precoating plates with fibronectin also eliminated the interfering activity, but since the clustering effect of pertussis toxin was also inhibited, this method could not be used.

With the above-described improvement of the CHO microplate pertussis NT, better results can be generated by analyzing serum samples from clinical trials involving pertussis vaccine; samples with otherwise interfering (“toxic”) activity may be analyzed.

The improved method has been used in a clinical trial at Statens Serum Institut to test the efficacy of a novel vaccine containing acellular pertussis toxoid, diphtheria toxoid, tetanus toxoid, and a new inactivated poliovirus vaccine produced without materials of animal origin and free of antibiotics.

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