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Antiviral Activities of Amniotic Fluid (41295)

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Abstract. Because viral infection in pregnancy can be a threat to the developing fetus, the identification and quantification of any protective factors operating on behalf of the offspring may be important. We have, therefore, assayed human amniotic fluid for factors influencing viral growth and have found that such fluid does not contain substances with an interferon-like effect, but does contain dialyzable components which will inactivate fibroblast interferon. Furthermore, amniotic fluids tested did not contain virus neutralizing activity as determined using vesicular stomatitis virus, but did contain specific antibodies against herpes simplex virus and cytomegalovirus.

Amniotic fluid (AF) has been shown in the past to inhibit the growth of various bacteria (1–4). Its antiviral properties, however, are less well understood although virus specific antibodies have been identified in AF specimens (5–7).

In light of the potential for certain viruses to spread to the fetus, it seems useful to better understand the antiviral properties of AF. To this end we have assayed a number of AF specimens, uniformly taken from women who required amniocentesis and genetic counseling and whose pregnancies had not yet reached the 21st gestational week. We have studied these fluids for interferon-like substances, interferon inactivators, vesicular stomatitis virus-neutralizing activity, and virus-specific complement-fixing antibodies.

Methods. *Patients.* Patients were recruited from local obstetricians. Any patient requiring amniocentesis and genetic counseling was considered a candidate for the study. Of the 51 patients included in the study, 45 required amniocentesis because of maternal age, 4 because a previous child had an anomaly, and 2 because the mother had a sibling with Down's syndrome. The ages of the mothers ranged from 35 to 45 years except for the 6 mothers who had a sibling or earlier child with an anomaly. The ages of these 6 mothers were 21, 24, 25, 27, and 30 years, respectively. All pregnancies were between the 10th and 20th weeks of gestation when amniocentesis was performed.

Amniotic fluid specimen. Amniotic fluid specimens were refrigerated and sent immediately to the laboratory for processing. Upon receipt AF were centrifuged at 1000 rpm for 20 min to remove cells and solid debris. The supernatant was then harvested and frozen at −80° until use.

Of the 51 specimens studied 49 had normal karyotypes, and 2 had abnormal karyotypes (one with Down's syndrome and one with a possible mosaicism).

Serum. Blood was taken from a forearm vein by venipuncture, allowed to clot, rimmed, and centrifuged at 1000 rpm for 10 min. The serum was harvested and frozen at −80° until use.

Interferon assays. Interferon assays were performed as previously described (8). An interferon standard calibrated against NIH reference standard G-023-901-527 was employed in every test.

Vesicular stomatitis virus-neutralizing activity. Virus-neutralizing activity was assessed using vesicular stomatitis virus (VSV). To perform the assay, AF were serially diluted in microtiter transfer plates (Microbiological Associates, Baltimore, Md.). Diluent consisted of L-15 tissue culture media containing 250 u/ml penicillin, 150 μg/ml streptomycin, 90 μg/ml arginine, 30 μg/ml glutamine, 1 mg/ml dextrose, and 37.5 mg/100 ml of $NaHCO_3$. After dilution, an equal volume of diluent containing approximately 100 $TCID_{50}$ of VSV was added to each well and incubation was performed for 30 min at 37°. At the completion of this incubation the virus AF mixture contained in the transfer plate well was discharged

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through pores in the base of each well into a microtiter well containing confluent foreskin fibroblasts (FF cells) and 0.05 ml of diluent. The plate was sealed and incubated until the virus titration performed as a part of every test demonstrated 100 $TCID_{50}$ of virus was present. The plates were then read by light microscopy. The neutralizing titer was considered the last dilution of amniotic fluid demonstrating a 50% inhibition of viral CPE.

Interferon inactivators. Interferon inactivators were sought as previously described (9). One part crude interferon was mixed with 3 parts of AF. Incubation was then carried out for 24 hr at 37° in a water bath. Concurrently an identical quantity of the same interferon solution was incubated in diluent as noted above. At the conclusion of the incubation all interferon samples were assayed for bioactivity. Residual activity in amniotic fluid was calculated as follows:

$$\text{residual activity} = \frac{\text{interferon activity in amniotic fluid}}{\text{interferon activity in diluent}} \times 100.$$

Preparation of virus induced leukocyte interferon and polynucleotide induced fibroblast interferon have been described in the past (10).

Antibody determinations. Measurements of antibodies and immunoglobulins were performed on lyophilized specimens of amniotic fluid which were reconstituted to 1/10th of their original volume. Measurements of IgG, IgM, and IgA were determined by radial immunodiffusion (11) and read at 48 hr. Complement-fixing antibodies were measured on both maternal serum and AF which had been concentrated as noted above. Antigens from herpes simplex virus and cytomegalovirus (CMV) were purchased from Microbiological Associates. Reference sera were purchased from the same source. Antibodies were measured in a microtiter assay (12) by means of the Laboratory Branch complement fixation tests of the Center for Disease Control (13). All specimens of amniotic fluid found to contain antibodies were retested on at least one occasion to confirm the presence of these agents, unless the specimen was exhausted in the initial assay.

TABLE I. THE EFFECT OF 24 hr INCUBATION AT 37°C IN AMNIOTIC FLUID ON HUMAN INTERFERON

	Interferon titers			
	Fibroblast interferon		Leukocyte interferon	
Specimen No.	AF titer	Control titer[a]	AF titer	Control titer
1	40[b]	160	160	160
2	40	160	160	160
3	40	160	160	160
4	20	160	10	10
5	20	160	10	10
6	10	320	80	80
7	20	320	80	80
8	20	320	80	80
9	40	320	80	80
10	80	80	N.T.[c]	N.T.
11	20	80	N.T.	N.T.
12	20	40	N.T.	N.T.
13	20	40	N.T.	N.T.
14	80	160	N.T.	N.T.
15	40	640	N.T.	N.T.
16	80	640	N.T.	N.T.
17	80	640	N.T.	N.T.
18	80	640	N.T.	N.T.
19	40	160	N.T.	N.T.

[a] Interferon incubated concurrently and under identical circumstances except L-15 diluent was substituted for AF.
[b] Expressed as units/0.025 ml.
[c] Not tested.

Results. *Interferon activity.* A total of 29 specimens of AF were tested for interferon-like activity. None of these specimens were found to contain such activity.

Interferon inactivation. Nineteen AF specimens were tested for interferon inactivators. All but one of these specimens at least partially inactivated human fibroblast interferon. Thus, mean residual activity for fibroblast interferon incubated 24 hr in AF was 27.4% (Table I) with a range of 3 to 100%. This difference between fibroblast interferon activity in AF and in diluent was significant ($P < 0.01$ by Student's t test). Nine specimens of AF known to contain inactivators of fibroblast interferon were also tested for ability to inactivate type I human leukocyte interferon, but in every case, leukocyte interferon was resistant to inactivation. The difference in residual activity between the two interferons was significant ($P < 0.01$). The biological charac-

teristics of the fibroblast interferon inactivators were further defined in two specimens of AF known to contain inactivators as described above. When interferon was incubated in these specimens at 4° rather than 37°, no consistent adverse effect on bioactivity could be shown. Furthermore, when incubation was performed immediately after addition to the AF, no loss of bioactivity occurred.

Dialysis for 24 hr against 100 vol of phosphate-buffered saline removed the inactivator from both specimens.

Vesicular stomatitis virus-neutralizing activity. Twenty-six specimens were tested for ability to neutralize vesicular stomatitis virus and none contained any neutralizing activity.

Antibody determinations. The mean concentration of IgG in 26 specimens of AF was 21.9 ± 9 mg/100 ml (standard deviation, SD) while the mean IgA concentration was 0.9 ± 0.8 mg/100 ml (SD). IgM was not detected.

The 26 AF specimens assayed for immunoglobulins along with concurrently drawn maternal sera were also tested for specific complement-fixing antibodies to herpes simplex virus and cytomegalovirus. Of the 26 specimens of concentrated AF tested, there were 8 with detectable antibodies to herpes simplex virus, and 7 with antibodies to CMV. The relationship of maternal to AF antibodies related to the level of maternal antibody (Fig. 1). Thus, of the 8 instances where the antibody titer in maternal sera was 64 or greater to either herpes virus or CMV, 7 (88%) had the appropriate antibodies detected in the concentrated AF. In contrast, of the 21 instances where antibody was detected in maternal sera to either herpes virus or CMV, but in titers of less than 64, only 7 (33%) had the appropriate antibody found in the AF ($P < 0.01$).

Discussion. Since studies of antiviral activity in amniotic fluid have been less systematic than those of antibacterial activity, we have sought several factors influencing viral growth in AF uniformly taken before the 20th gestational week.

None of the 29 specimens of AF tested for interferon contained this type of activity suggesting that interferon is not a normal

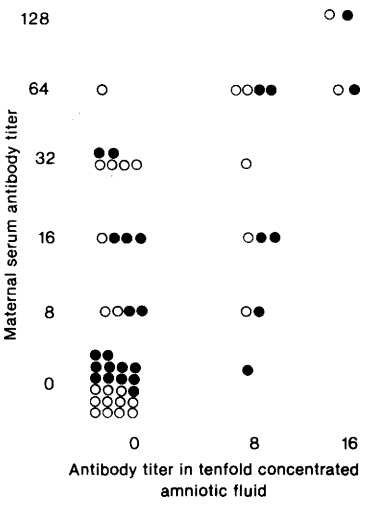

FIG. 1. Relationship between maternal serum and amniotic fluid complement fixing antibodies. Antibodies to herpes simplex virus indicated by solid circles and to cytomegalovirus by empty circles.

finding in AF and that future research looking for markers of intrauterine viral infection should include studies of the ability to detect interferon in these situations as a possible diagnostic aid.

It is theoretically possible that interferon could be used, possibly by intraamniotic injection to either treat or protect the fetus from viruses infecting the mother. If this potential is realized, the presence of substances in AF which inactivate fibroblast interferon but seem to have no effect on leukocyte interferon would likely give the latter a therapeutic advantage. Fibroblast interferon inactivators in AF were found to be dialyzable substances which are both temperature and time dependent. In this regard, AF inactivators are similar to those we have described in other body fluids (8).

The presence of such inactivators in AF may also diminish the chance of detecting interferon and may have contributed to our inability to find this antiviral protein; however, these studies have shown that leukocyte interferon is not affected by these inactivators and hence should not be obscured by their effects. Furthermore, the inactivators took rather lengthy incubation periods to demonstrate and if fibroblast interferon were being actively produced in significant quantities some residual anti-

viral activity might have been found. The chance of recovery of fibroblast interferon was enhanced by prompt refrigeration of the AF samples as these studies have shown the inactivators are not effective at 4°.

By utilizing vesicular stomatitis virus as a test virus and exposing this agent to various specimens of AF we sought neutralizing factors contained in this fluid.

The purpose of these studies was to seek previously undefined substances other than traditional antibodies which might have deleterious effects on a broad range of viruses, and whose presence might not require previous contact with the test virus. As we knew virus-neutralizing antibodies could be present in AF (15) and as we knew the presence of neutralizing antibodies required prior immunological experience with the virus in question or with a related virus, we selected VSV for these studies. We felt humans were unlikely to have had prior experience with this agent and hence would not have specific VSV-neutralizing antibodies. Antibacterial substances previously found in AF include factors other than antibodies (16) and hence provided us with a precedent which we hoped to apply to viruses; however, we could not identify any VSV-neutralizing substances in AF. We conclude AF does not contain any viral-neutralizing substances with broad effects against all viruses including VSV. Our results contrast somewhat with those reported by Pacsa and Pejtsik (17) who used a very similar assay to ours except they used herpes virus and poliovirus as test agents. They found 7 of 44 specimens contained virus-neutralizing activity to either one or both test viruses and the activity they detected did not appear to be mediated by an immunoglobulin of the IgG class. It is possible by employing a dissimilar virus we have obtained different results.

Many other investigators (5–7, 18–22) have measured AF immunoglobulin levels and such determinations were repeated here only for the sake of completeness. The mean IgG level of 21.9 mg/100 ml and the mean IgA level of 0.9 mg/100 ml found in our study is in the range reported in the literature. Many authors, including ourselves, have not found IgM in AF (19), however, recently this immunoglobulin has been found in AF using either greater concentration or more sensitive methods (20–22).

Specific viral antibodies have also been reported present in AF in previous investigations (5–7); however, the studies reported here have extended earlier investigations by uniformly measuring antibody levels during early pregnancy (before 20 weeks), by using paired specimens of maternal serum and AF, and by concentrating AF 10-fold prior to assay. The results obtained here verify that specific antibody can be found in AF and its presence relates to the level of maternal antibody. This is in agreement with the data of Sutcliffe and Brock (23) who, although not actually studying immunoglobulin, concluded that after the 10th week of gestation the major soluble protein in AF is primarily of maternal serum origin. Other investigators including Cederqvist *et al.* (22) who specifically studied AF gamma globulins and Johnson *et al.* (23) have reached similar conclusions.

Lastly, Bradley *et al.* (26) have recently noted the level of herpes simplex-neutralizing antibody in AF correlates with the level of similar antibody in maternal serum.

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