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THE DEVELOPMENT OF A DEFENSE SYSTEM AGAINST COXSACKIEVIRUS B5 INFECTION IN SUCKLING MICE

by

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ABSTRACT

There are many viruses that are able to infect the alimentary tract of man. Little is known, however, about the mechanism of infection itself or the pathophysiology of the gut during infection. The research reported here is concerned with the differences in susceptibility among suckling mice of various ages innoculated by the intraperitoneal and intragastric routes.

Since the normal mode of entry of many viruses to the gut is via the oral route, Coxsackievirus B5, a human enterovirus which does attack this way, was utilized. It is a non-tumor producing RNA virus that has been shown to act similarly in the mouse and human.

The virus was pooled in HeLa cell cultures and titered by a plaquing assay in the same cell cultures. CD-1 mice, 10, 14, 18, and 22 days old were infected either orally or intraperitoneally with 5.0×10^{10} (10 day old animals) and 1.0×10^{9} plaque forming units per animal. Dissections were done at 1 and 3 days post infection with samples of the blood, heart, liver, and gut being taken from each animal. Each sample was titered individually and the data presented as an average of six samples.

As a result of previous work, it is known that the gut of a newborn mouse isn't able to decrease the concentration of the infecting dose and therefore provides no defense against an enteric infection with Coxsackievirus B5. In contrast,

mature mice are able to reduce the amount of viral dissemination across the gut as well as inhibit replication after absorption has occurred.

The results of this study indicate that there is a double barrier system developing in suckling mice that is involved with and directly related to the gastrointestinal tract. The first part of this defense is the inhibition of penetration of virus across the gut when the primary site of infection is the intestinal mucosa. This mechanism develops sometime around 20 to 22 days after birth. At about 16-18 days of age, suckling mice that were challenged intragastrically are able to stop active replication and initiate clearance of virus from the systemic circulation.

There are many factors that might contribute to the marked decrease in susceptibility with age of suckling mice. Some of these or possibly a combination of these factors might explain the defense mechanisms described above, but to date, the chemistry or mechanical functioning of the gastrointestinal barrier to enteric viral infection is unknown.

Introduction

Viral agents commonly infect the alimentary tract of man. The outcome of these infections is either subclinical or clinical, with a wide variety of symptomatology. In particular, enteroviruses have been shown to produce clinical infections of the gastrointestinal tract in humans (Sheehy, et al., 1964; Pelon, 1965; Kibrick, 1964; Rivers, 1959; and Lenette, et al., 1969). Even though there are many viruses that are known to cause gastrointestinal tract infections, little is known about the pathophysiology of the gut during infection.

Coxsackie viruses of the B group were discovered in 1949 by Melnick, Shaw, and Curnen. They were shown to cause tremors and spastic paralysis in newborn mice. The virus was also proven to have a very strong effect on the pancreas and liver, as well as the gut. It is an RNA virus of approximately 2×10^6 molecular weight with a lipid envelope. It is resistant to a pH range from 2.3 to 9.4 and is stabilized by divalent cations such as Mg^{++} rendering it quite heat stable.

Infection by the B group of Coxsackievirus can result in any of the following disorders: paralysis, aseptic meningitis, herpangina, pleurodynia, pericarditis, myocarditis, meningoencephalitis, pneumonia, hepatitis, and possibly diabetes (Baker, 1971; Plummer, 1965; and Kibrick,1964).

As might be expected, there are many parameters involved in determining susceptibility to a viral infection in an animal model. Certain strains of mice are more susceptible than

others to Coxsackievirus B infection. Bang, _et_ _al_. (1961) postulate that this is due to genetic variations among strains. Even within pooled litters, differences are evident (Sulkin, _et_ _al_., 1951). Specific virus strains are known to differ in degrees of neurotropism and pancreotropism as a result of how and in what type of cell culture they were passed.

Sigel (1952) and Bang, _et_ _al_. (1961) have studied age versus susceptibility in viral infections involving the intraperitoneal route only. They have shown a definite decrease in susceptibility with age but it is really inconclusive since an unnatural mode of innoculation was used.

Goldblum (unpublished data) in a somewhat cursory study of many viruses, reported that by the tenth day in life, mice reached a minimum in susceptibility to a viral challenge.

Some of the proposed theories for age specific susceptibility are: 1) delayed or impaired antibody formation (Dalldorf, 1955), 2) immunologic tolerance (Hotchin, 1960), 3) rise in interferon level (Heinberg, _et_ _al_., 1964; and Rytel, 1964), 4) sex hormones (Berkovich, _et_ _al_., 1965, 1967), 5) natural tissue barriers like the brain blood barrier (Sabin, 1941; Sigel, 1952; and McLaren, _et_ _al_., 1959), and 6) availability of virus receptor sites (Kunin, 1962). Actually no one of the above factors is meant to be a complete explanation.

As mentioned above, the mode of infection, whether by the intraperitoneal, intragastric, subcutaneous, or intracerebral route has been shown to have a distinct effect on susceptibility

(Johnson, 1955; Kaplan, et al., 1951; and Bang, et al., 1961). Enteroviruses enter humans via the oral route.

The amount of the dose is quite important too, since at a high enough dosage, animals of any age might appear to be equally susceptible.

Sex and environment can have a great deal to do with the course of a viral infection. Specifically, male mice seem to be more susceptible than females (Berkovich, et al., 1965, 1967; Gevauden, et al., 1965). As far as living conditions are concerned, it has been shown that animals kept at 4° C show a significant increase in susceptibility in contrast to those kept at 25° C (Boring, 1956; Gevauden, et al., 1965, 1966). Also, mice demonstrate distinct diurnal rhythyms of some major hormones which implies that light and dark periods would have to be regulated.

Finally, the sampling size would have to be large enough so that differences in susceptibility between animals of the same litter would not statistically bias the results.

There has been very little research done in the area of oral murine infections. In a study by Kaplan and Melnick (1951) a great difficulty in getting the animals to swallow the entire dose was reported. Maximovich, et al., (1966) inoculated one to two day old mice with an aerosol but was unable to get a reproducible dose every time. Von Magnus (1950) introduced a drop of encephalomyelitis virus of mice into the by mouth and then pulled on the tail in an effort to deliver the dose. Other studies have involved force feeding

after chloroform treatment but this was also a method that was quite unreliable in regards to the accuracy and consistency of the dose.

Newborn mice are known to be equally susceptible to enteric infection by Coxsackievirus B5 via either the intragastric or intraperitoneal routes. This means that the virus titer at a sufficient time after infection for absorption to occur, is essentially the same in all organs assayed regardless of how the virus was originally administered. However, in mature animals (at least 35 days old), there is a marked difference in titer between those animals infected intraperitoneally and those infected intragastrically with the latter group showing the lower titers. From this data it can be concluded that a directional barrier exists in the gastrointestinal tract of mature mice inhibiting the dissemination of virus from the mucosal side of the gut to the peroral side (Shadoff and Loria, unpublished data).

The purpose of this research was to determine the point in the life of mice when this effective defense system to fense a viral gastrointestinal infection develops. Extrapolation of this data to the human clinical experience may not be totally justified, yet valuable information concerning the nature of the infection might prove to be helpful in the study of similar human situations.

Since the normal mode of infection by enteroviruses is via the oral route, susceptibilty comparisons would have to be done using this method of infection. Previously, little

has been reported about the differences in susceptibility of newborn or suckling mice to oral and intraperitoneal infections with a human virus. One study showed that Coxsackievirus B infection in the newborn mouse is usually lethal whereas mature animals are only mildly affected (Kaplan and Melnick, 1951). It is therefore of specific interest to determine the differences in susceptibility between intragastric and intraperitoneal infections in both newborn and suckling mice.

Coxsackievirus B5 and the mouse as an animal model were chosen because of the following.

1. The virus is known to cause enteric infections.
2. Coxsackievirus B5 has been used before in experimental mouse infections.
3. Mice are easily handled because of their small size.
4. There is a definite correlation between man and the mouse during an infection with Coxsackieviruses of the B group (Rytel, *et al.*, 1971).
5. Tissue culture techniques for production and titration of the virus are available.

Materials and Methods

Virus

The Grenier strain of Coxsackievirus B5 was used in this experiment. It was originally isolated in monkey kidney tissue cultures from patients with aseptic meningitis. Identification of the agent as Coxsackievirus B5 was confirmed by neutralization tests using antiCoxsackievirus B5 rabbit sera obtained from Microbiological Associates, Inc., Maryland. The virus was then passed four times in HeLa cells to obtain a sufficient volume of adequate titer for the experiments. It was stored at -70° C in a Revco freezer and diluted in the same minimum essential medium with Earle's salts that was used in the tissue culture work.

Animals

CD-1 mice from Charles River Breeding Laboratories, Massachusetts, were used. They were obtained as 15-17 day untimed pregnant females. Upon arrival, the animals were placed in individual disposable cages (Carworth, New York) and given Purina Lab Chow *ad libitum*. They were allowed at least four days for adaptation to their new surroundings before an experiment was initiated. The animal room was maintained at a temperature of approximately 23° C. The animals were housed in a windowless room which was continuously being vented by a large exhaust fan and was illuminated for ten hours each day from 9:30 A.M. to 7:30 P.M. by incandescent

bulbs on an automatic timing system.

Litters consisted of 9-11 animals. In order to minimize the differences in susceptibility among suckling mice, more litters than were needed were infected with the same dose at each time interval. Sampling was then random among animals infected at the same time. When the peroral route was being compared with the intraperitoneal route of infection, innoculations of animals at a specific time in their life all took place on the same day and under identical conditions. (All the 10 day old animals were infected on the same day, etc.)

Infection and Sampling Procedures

Animals were infected by two routes, intragastric and intraperitoneal, at four different ages, 10, 14, 18, and 22 days old. At each age, five litters were infected intraperitoneally, five orally, and five were given control doses of minimum essential media with Earle's salts, half orally and half intraperitoneally. The dose for the 14, 18, and 22 day old animals was 1.0×10^9 plaque forming units (pfu) per animal and the dose for the lo day old animals was 5.0×10^{10} pfu per animal.

Intraperitoneal injections were administered by inserting a sterile 30 gauge, 1.5 inch long needle (Vita Needle Company, Massachusetts) subcutaneously, parallel to the femoral vein, and then into the peritoneal cavity. The infection was in 0.1 ml portions and loaded in 1 cc tuberculin syringes.

Intragastric infections were carried out using an intubation technique. An Intramedic P. E.-50 (Clay Adams, New York) polyethylene tube, 5.0 cm long, mounted on a Clay Adams Type A tubing adapter attached to a 1 cc tuberculin syringe was used. The tube and adapter were sterilized in boiling water for thirty minutes before use. The tube was guided down the esophagus using the natural swallowing reflex of the animals and the dose was delivered. Using India Ink as the inoculum, it was observed that this length tube left the injected solution in the gastric lumen. This method could therefore be relied on to give consistently reproducible results of oral infections.

If damage was done as a result of the infection, animals would usually die within a day and would not be used in the experiment. Likewise, animals dying within a day from the shock of either of the two techniques would be discarded from the experiment and samples of their tissues would not be used.

Dissections were done at 1 and 3 days post infection. Twelve animals from orally infected groups, twelve from intraperitoneally infected groups, and twelve control animals were dissected at each of the age groupings (10, 14, 18, and 22 days old), half at 1 day post infection and the other half at 3 days post infection. Those animals dissected were randomly selected from a large group of inoculated animals.

Animals were killed by decapitation and the blood was drained into sterile 5.0 ml sample vials. At this time it was diluted 2:1 with sodium heparin to prevent coagulation

and quick frozen in a dry ice ethanol mixture. Eventually the blood was thawed and titered for virus content in the above heparinized form. Any further dilutions were done with sterile Hank's solution containing 25,000 units each of Penicillin, Streptomycin, and Amphotericin per 100 ml.

Heart, liver, and gut from infected and control animals were quickly removed and cleaned free of all fat and blood vessels. They were then thoroughly washed in ice cold phosphate buffered saline. Hearts and livers were individually stored in sterile screw cap sample vials and quick frozen in a dry ice ethanol mixture. The gut was cut open longitudinally, starting at the stomach and continuing distally to the rectum and flushed of all contents possible with ice cold phosphate buffered saline. This was done to assure that virus in the gut, when assayed, would only be that virus absorbed in the tissue itself and not from any food that was moving through the gut. The cleaned gut was then stored and frozen exactly as the hearts and livers had been.

Assay Technique

Media, serum, and other tissue culture materials were obtained from Flow Laboratories Inc., Maryland, and Microbiological Associates Inc., Maryland.

HeLa cells (Human Cervical Carcinoma) were used throughout the experiment. Normal tissue culture techniques were employed for the upkeep of the continuous HeLa cell line and for the plaque assay described below.

The plaque technique for assaying titers of enteroviruses

as described by Melnick and Wenner (1969) was used with the following modifications. Monolayers of HeLa cells were grown to confluency in sterile 60 mm plastic disposable Petri dishes instead of bottles. The cell sheets were examined before the assay was begun to assure that a continuous monolayer was being used.

The samples were thawed for preparation. This involved homogenization with mortar and pestle followed by a 10% dilution with sterile water (twice distilled) containing 25,000 units each of Penicillin, Streptomycin, and Amphotericin per 100 ml of solution. The homogenates were then frozen and thawed three times overnight in sterile 15 or 50 ml plastic centrifuge tubes. The mixture was then centrifuged at 1000 RPM for 15 minutes and the supernatants containing the freed virus were used as plaquing innoculums. Further ten fold serial dilutions were done with the same Hank's solutions used in blood preparation. All samples at every step of preparation were stored at -70° C in a Revco freezer.

An inoculum of the homogenated virus containing tissue was added to the cell sheet and allowed to absorb for 45 minutes at 37° C in a carbon dioxide incubator. Four ml of the following media agar overlay at 50° C was then added to the plates and allowed to cool at room temperature.

<u>Agar Media Formula</u>

2x Earle's minimal essential medium containing

2% glutamine	47.3 ml
$NaHCO_3$ 7.5%	2.7 ml

Fetal calf serum (inactivated)	5.0 ml
50% $MgCl_2 \cdot 6H_2O$	1.0 ml
Penicillin and Streptomycin (10,000 units/ml)	1.0 ml
Amphotericin (10,000 units/ml)	1.0 ml
Difco Agar 3%	42.0 ml
	100.0 ml

The plates were then inverted and incubated for 40 hours.

After incubation, the plates were stained with 0.5 ml of 0.1% neutral red which was prepared in isotonic phosphate buffered saline at pH 7.2. The plates were then incubated for 3 hours in the dark and finally plaques were counted as white holes in the red cell sheet.

Results

CD-1 mice, ages 10, 14, 18, and 22 days old, which were infected with 1.0×10^9 pfu per animal (14, 18, and 22 day olds) and 5.0×10^{10} pfu per animal (10 day olds) were sacrificed at 1 and 3 days post infection and then dissected. Table 1 on page 15 gives virus titers in pfu per gram of tissue for heart, liver, and gut, and pfu per ml of blood as their corresponding log values. Each value is an average value for titrations done on six separate samples. The average deviation for each value shown was never more than 0.6 log units on either side of the mean. Therefore a difference of at least two log units between any two means would imply a significant difference between these means.

It is assumed that the virus recovered actually represents the amount of the virus in the tissue itself since fat, blood vessels, and in the case of the gut, intestinal contents were removed. Furthermore, the rinses in ice cold phosphate buffered saline should wash out any macromolecules loosely attached to the tissue surfaces that would be capable of binding virus particles.

At 1 day post infection in 10 day old animals, it is quite clear that there is no difference in virus content with respect to route of infection in all organs sampled. The only noticeable difference is that the bloods of 10 day old animals contained virus concentrations which were up to a hundred fold lower than the concentrations found in the liver and gut regardless

of the mode of infection. This phenomenon repeats itself at 1 day post infection throughout the experiment. At 3 days post infection in 10 day old animals, replication has occurred in animals infected by both routes.

For 14 day old animals, there is again no significant difference in titers with respect to the intragastric or intraperitoneal route at 1 day post infection. However, at 3 days post infection, virus concentrations in tissues of those animals infected intraperitoneally are indicative of active replication whereas there is a definite drop in titer of approximately 3 log units in those animals challenged intragastrically.

This drop in virus titer, which suggests some mechanism of clearance, reaches a maximum in 18 day old animals. It can be seen that at 3 days post infection there is consistently up to a hundred thousand fold difference in virus content per gram of tissue or ml of blood between modes of infection in all samples assayed. Therefore, replication is taking place when the site of viral penetration is the serosa and clearance is taking place when the mucosa is the primary site of infection.

A difference in virus titers of the organs assayed between intragastrically and intraperitoneally innoculated animals at 1 day post infection indicates an immediate barrier to the dissemination of virus. In the 10, 14, and 18 day old animals there is no such difference but in 22 day old animals there is a 2 log difference. This gastrointestinal barrier is inhibiting the penetration of Coxsackievirus B5 into the circulation and thereby causing an effective reduction of

the infecting dose.

Figures 1 and 2 on page 16 illustrates these titer differences.

Table 1. Sample titrations.

Age		IP	Oral	IP	Oral
		1 day post infection		3 days post infection	
10	Blood	6.70	6.24	9.87	9.30
	Heart	7.47	7.39	9.33	8.52
	Liver	8.34	8.10	9.26	9.00
	Gut	8.45	8.35	9.59	9.69
14	Blood	5.58	3.51	7.58	4.00
	Heart	6.59	4.65	8.04	4.24
	Liver	7.54	5.18	8.15	5.27
	Gut	6.96	6.59	8.19	5.27
18	Blood	5.48	5.61	6.54	2.70
	Heart	5.41	4.67	8.02	2.24
	Liver	6.84	5.78	7.98	3.17
	Gut	6.38	5.13	8.19	3.20
22	Blood	5.46	3.00	7.18	2.08
	Heart	6.09	4.24	7.61	3.75
	Liver	6.93	4.59	8.07	3.17
	Gut	7.15	5.14	9.12	3.40

All values above are $\log_{10}$ values of plaque forming units per gram of tissue or ml of blood. Each is an average of six samples assayed.

Fig. 1
Log_{10} pfu of 18 day old animals 3 days post infection

IP- Intraperitoneal

IG- Intragastric (oral)

Fig. 2
Log_{10} pfu of 22 day old animals 1 day post infection

Discussion

A previous study established the increased susceptibility of the newborn mouse to infection via the oral route with a human enterovirus, Coxsackievirus B5. It showed that the alimentary tract of neonatal mice provided no barrier against the penetration of virus into the systemic circulation. Immunoflourescent staining indicated viral antigens localized in the epithelial cells of the intestinal mucosa.(Shadoff and Loria, unpublished data). This result supports Sabin's hypothesis that it is the epithelial cells of the intestine that are primary sites for enteroviral attachment and replication rather than Peyer's patches and other lymphoid tissue of the gut as has been suggested by Bodian.

In contrast, the gut of the mature mouse does possess an effective defense mechanism against an enteric Coxsackievirus infection. There appear to be two separate mechanisms operating; the first is a barrier that inhibits the dissemination of virus from the mucosal side of the gut into the circulation; the second is a virus clearing system that is in operation when the intestinal mucosa is the original site of penetration. When the penetration is across the serosa however, there is a significant delay in clearance of virus from the system.

It appears that the clearance mechanism develops first. The maturation of the clearance system begins in animals at sometime between 14 and 16 days of age. It is not until the animals are 18 to 20 days old, however, that the system attains

maximum efficiency. It is clear from the data that the two mechanisms develop independently of each other and at different times in the life of the animal. The specific barrier to dissemination begins its development about four days later in animals 22 days old. Previous to this time in the animal's life, it had no means of reducing the infecting dose by either route.

The marked susceptibility of the newborn mouse to Coxsackievirus infections seems to be related to the morphological development of the gut. It has been reported by Lecce (1972) that the gut of a neonatal mouse is capable of non-selectively absorbing a wide variety of macromolecules including many different albumins. Of particular interest is the fact that gamma globulins can cross the gut and enter the circulation throughout the weaning period. It isn't until 18 to 21 days after birth that a closure of the gut takes place. Also at this time, there is a change in the structure of the microvilli of the intestinal mucosa from a broad, rounded appearance to the long pointed microvilli that are evident in mature adults (Overton, 1965). At the beginning of the third week of life, there is an increase in the turnover rate of epithelial mucosal cells. This might possibly account for a simple washing out effect of virus containing lining cells.

Along with these morphological changes are various functional developments that would tend to decrease susceptibility with age. There is a rapid increase in concentrations of various hydrolytic enzymes such as phosphatases as the animal

is weaned (Moog, 1950, 1951, and 1967). Since the Peyer's patches and other lymphoid structures of the gut are undeveloped until the suckling period is over, there exists no general mechanism for the combat of a viral infection (Baintner and Veress, 1970).

As mentioned above, large protein molecules like globulins, which are usually highly antigenic in nature, can pass through the gut. The molecular weight of gamma globulin is 3.85×10^5 and that of Coxsackievirus B5 is 2.0×10^6. Therefore it is possible that a similar mechanism of penetration takes place but just how open the gut is to particles of molecular weight two million is not yet known. However, the fact that in 10 day old animals there is no difference in susceptibility between routes of infection seems to strenghthen this hypothesis. that the porous gut of a suckling mouse is capable of allowing Coxsackievirus virions to move freely into the circulation from within the gut.

Even if the virus couldn't penetrate the gut by the above method, mechanical passage is always another possibility. Along with these factors, is the concept of undifferentiated intestinal cells as compared to those of adults which are quite specialized. This overall immaturity of the gut would tend to make it the ideal site for primary infection, increasing the susceptibility of the host.

If the morphological development of the newborn mouse is similar to the newborn rat, there are also no plasma cells until the fifth or sixth week of life (Bridges, et al., 1959).

Since the secretory piece of IgA (Immunoglobulin A) but not the entire antibody is synthesized by newborns and since it is known that the entire molecule is necessary to have any effect at all on antigens, it is evident that there is no general help afforded by any local antibodies such as IgA so early in life. There has also been no evidence of any considerable levels of IgG or IgM in the sera of neonatal rodents.

Hormones, or lack of them early in life, might relate to the increased susceptibility of newborns. Estrogens cause increases in mitotic activity and thereby increase turnover rates of the cells of the epithelial mucosa (Bullough, 1966). Cortisone is known to speed up the differentiation and development processes of the gut but on the other hand it also causes increases in susceptibility to Coxsackievirus B infections in adults.

In conclusion, there are many factors which could increase susceptibility in suckling mice. Some of these, which change considerably with age, could also be construed to explain the decrease in susceptibility as the mouse matures. To date, it is not known by what mechanism or combination of mechanisms that the dual defense system to a viral infection described in this work operates. However, the data presented here have at least elucidated the problem of when in the life of a mouse or possibly even in that of a human, there develops some kind of defense against enteric viral infections.

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