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RELATIONSHIP OF COLD UPON THE BIOLOGICAL PROPERTIES PECULIAR TO INFLUENZA VIRUS AND ITS CAPACITY FOR INVASIVENESS AND MULTIPLICATION

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Prepared under Contract AF41(657)-350 by
T. G. Metcalf, Department of Bacteriology
University of New Hampshire,
Durham, New Hampshire

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RELATIONSHIP OF COLD UPON THE BIOLOGICAL PROPERTIES PECULIAR TO INFLUENZA VIRUS AND ITS CAPACITY FOR INVASIVENESS AND MULTIPLICATION

Project personnel have been studying the microbiology of the arctic ground squirrel in the nonhibernating state. The first objective was to determine whether, under normal conditions, Alaskan ground squirrels excrete animal or bacterial viruses from the gastrointestinal tract.

A freshly passed fecal specimen was obtained and a thick emulsion prepared by mixing the sample with an equal volume of water. The emulsion was centrifuged at 12000 RPM for 30 minutes at 5° C and the supernate was removed and used in the examination for virus.

The search for bacterial virus was carried out with five different strains of Escherichia coli. Four of the five strains were isolated from the ground squirrels; the fifth was E. coli B, the mutant host cell used with coli phages. A volume of supernate was added to a layer of the host cell culture in a Petri dish, using the Dulbecco agar overlay technic to produce plaques in the presence of virus. Eighty-one separate samples were collected at various times from 25 ground squirrels and examined for bacterial virus. To date no bacterial virus has been found in any of the ground squirrels examined. The examinations are presented in Table I.

The search for animal viruses was conducted with three cell cultures carried in continuous cultivation, the LLMK2 culture of monkey kidney, HeLa and Human Amnion. All three cell lines have been cultured in monolayers in 160 ml bottles in order to gain the greatest likelihood of isolation. A 10 ml volume of supernate, containing 500 units of penicillin, 500 micrograms of streptomycin and 20 units of mycostatin per ml, was allowed to remain at room temperature for one hour. The sample was then added to a monolayer and allowed to remain in contact for one hour. At the end of this interval the sample was removed, the monolayer rinsed with balanced salt solution and 10 ml of appropriate maintenance medium added. The monolayers were incubated at 36° C until the controls showed extensive degeneration or cytopathogenic effects were noted in the test bottles.

Sixty-six individual samples collected from 24 ground squirrels at various times have been examined. Thirty-four samples representing 21 animals have been examined in HeLa cultures. Thirteen samples collected from 12 animals have been cultured in Human Amnion monolayers. Nineteen samples from 15 animals have been examined in monkey kidney monolayers. No definite isolations of virus have been obtained as yet. The examinations are shown in Table II.

TABLE I

Examination of Alaskan Ground Squirrels for Evidence of Excretion of Bacterial Virus

Trial	Animals Represented in Pool	Virus Isolation	Cumulative Totals Animal	Trials
1	1	negative	1	5
2	1-12-13-20-25	"	2	4
3	11-12-13	"	3	4
4	3-13-21	"	4	1
5	20-25	"	5	1
6	13-14-22	"	6	3
7	16-22-12-13-14	"	7	3
8	1-20-25-29-19-18-6-28-12-13-14	"	8	-
9	2-20-25-4-5-29-19-3-14	"	9	-
10	2-11-7-23-28	"	10	1
11	22-16-11-12-13-7	"	11	4
12	6-18-22-20-25	"	12	7
13	18-11-7-6	"	13	8
14	2-3-12-14-22	"	14	6
15	12-13-14	"	15	1
16	1-6-20-22	"	16	3
17	1-2-10-20	"	17	1
18	3-15-17-24	"	18	3
			19	2
			20	7
			21	1
			22	6
			23	1
			24	1
			25	5
			26	-
			27	-
			28	2
			29	2

TABLE II

Examination of Alaskan Ground Squirrels for Evidence of Excretion of Animal Virus

Animal	Cell Cultures Used for Virus Isolation		
	HeLa	Amnion	Monkey Kidney
1	ΔΔ	0	-
2	Δ	-	-
3	Δ	-	1
4	ΔΔ	-	-
5	ΔΔ	0	1
6	ΔΔ	-	11
7	Δ	-	-
8	-	-	-
9	-	-	-
10	Δ	0	1
11	Δ	0	1
12	-	-	1
13	-	-	-
14	-	-	1
15	ΔΔ	0	1
16	ΔΔΔ	0	1
17	-	-	-
18	-	-	11
19	-	-	-
20	ΔΔ	-	-
21	Δ	0	11
22	Δ	0	11
23	ΔΔΔ	00	1
24	Δ	-	1
25	Δ	0	-
26	ΔΔ	-	-
27	ΔΔ	-	1
28	ΔΔ	0	-
29	Δ	0	-

Legend

Δ = trial in HeLa culture

0 = trial in Amnion culture

1 = trial in Monkey Kidney culture

The second objective was to determine the immunologic response of Alaskan ground squirrels in the nonhibernating state to subcutaneous and intraperitoneal injections of bovine gamma globulin. A 20% aqueous solution of the antigen was prepared and 0.5 ml introduced intraperitoneally into one ground squirrel. A subcutaneous injection was made in one animal with 0.5 ml of emulsified antigen. The emulsion was prepared by mixing equal parts of 20% bovine gamma globulin and Arlacel-Drakeol. The mixing was performed in syringes connected with double-hubbed needles.

Blood specimens were obtained by cardiac puncture from each animal at 0, 5, 10, 15, 21 and 30 days following injection. The blood specimens were processed for serum and the antibody content determined by means of tube precipitin tests. No antibody production could be shown at 30 days in either animal.

The need for a greater number of ground squirrels led to a consideration of establishing a breeding colony. The animals have been quartered in individual cages in a normal animal room with temperature and humidity controlled. The animals were sexed and eleven males added to cages containing eleven females. There have been two litters produced with five baby ground squirrels per litter. In addition it is believed that three more females are pregnant and will produce litters soon.

The breeding experiment was accomplished without fighting in any of the 11 cages. None of the animals has been injured in any way. The baby squirrels are being weaned by the mother and appear to be in excellent health. The animals have been maintained on a diet of Purina Laboratory Chow, fortified with carrots once a week and apples three times a week. Vitamins and wheat germ oil have been added to the diet from time to time and, most recently, a handful of sunflower seeds has been supplied once a week.

The inability to detect virus growth at 20° C in chick embryo raised the issue of neuraminidase activity at temperatures lower than 37° C. Accordingly, an Asian strain of influenza virus was combined with neuraminmucoid at 25° C and the release of neuraminic acid examined at intervals up to six hours. The results are shown in Table III. A progressive splitting-off of neuraminic acid occurred during the 6-hour period of examination.

The use of ovomucin as a substrate for neuraminidase was explored. One dozen fresh eggs were collected from the University Poultry Farm. The albumin content was separated from the yolk and chalazae, homogenized in a Waring Blendor and centrifuged for 30 minutes at 35000 RPM. The precipitate remaining after centrifugation was suspended in 0.15 N NaCl and stored at -70° C. Ovomucin substrate was combined with a preparation of influenza virus shown previously to possess enzymatic activity. The neuraminic acid

split off was measured by means of the thiobarbituric acid assay. A progressive increase in neuraminic acid was determined for the virus-substrate mixture up through a 6-hour incubation period.

TABLE III

Enzymatic Cleavage of Neuraminic Acid from Neuraminmucoid Substrate by Influenza Virus at 25° C During the Course of a 6-Hour Period

	Time (Hours)			
Blank	1	2	4	6
0.16*	0.19	0.23	0.34	0.51
0.13	0.21	0.21	0.38	0.50
0.14	-	-	-	-
0.14**	0.20	0.22	0.36	0.505

* Optical density determined in spectrophotometer at a wave length of 549 mμ

**Average of optical density values determined

DISCUSSION

Major research emphasis has been directed to background studies preparatory to the conduct of hibernation investigations. The first objective has been to determine whether the hibernator (Alaskan ground squirrel) excreted animal or bacterial virus under normal conditions. The negative findings for animal virus were obtained with monolayers sixteen-fold greater in area than the customary tube cultures. This allowed an eight times greater sample to be tested than would have been possible if the conventional two tubes had been used. Thus, the 17 separate samples examined in monkey kidney monolayers represented the equivalent of 136 conventional samples. When all three types of cell monolayers are considered, the 66 samples were the equivalent of 528 conventional samples. The examinations were conducted during a two-month period.

In spite of the sampling interval and number of examinations made, it is recognized that it is still possible for one or more of the ground squirrels to

be virus excretors. The impression obtained however, is that these animals are not carriers of the enteroviruses which it is planned to use in the next phase of the study. Consequently, the next phase of determining the response to controlled dosages of virus may now be initiated. The results of the antibody study were surprising. The antigen selected, the use of adjuvant and the route employed have been shown repeatedly to be productive of an immunologic response in other hosts. No known cause for lack of immunogenicity of the bovine gamma globulin can be given. This antigen has been supplanted by a bacterial suspension which is currently being used to study the immunologic response of the ground squirrels in the nonhibernating stage.

The demonstration of neuraminidase activity at 25° C indicated that the enzyme continues to function at temperatures lower than those of the human body although the amount of neuraminic acid split from substrate was less than the amount measured at 37° C.

SUMMARY

Alaskan ground squirrels have been examined for their excretion of animal and bacterial viruses. Eighty-one separate fecal samples from 25 animals yielded negative findings for bacterial viruses reactive against five strains of Escherichia coli. Sixty-six individual fecal samples from 24 animals failed to show the presence of animal viruses capable of causing cytopathogenic effects in HeLa, Human Amnion and the LLMK$_2$ strain of monkey kidney monolayers.

The significance of the findings for animal viruses is presented.

Two successful ground squirrel breedings were accomplished with five baby squirrels produced in each litter.

Neuraminidase activity of influenza virus was demonstrated at 25° C. The amount of neuraminic acid split from substrate by enzyme action at 25° C was less than that observed at 37° C. Ovomucin obtained from fresh hens eggs was prepared and shown capable of serving as a suitable substrate for influenza neuraminidase.