LASTING BIOLOGICAL EFFECTS OF EARLY ENVIRONMENTAL INFLUENCES*

III. Metabolic Responses of Mice to Neonatal Infection with a Filterable Weight-Depressing Agent

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Mice obtained from so-called "specific pathogen-free" colonies (SPF) and protected from contamination during the first 3 wk of their lives, are heavier at weaning time and become larger adults than mice of the same genetic stock raised under ordinary conditions of husbandry (1, 2). The weight curve of these SPF animals can be profoundly and lastingly depressed by contaminating them shortly after birth with a filterable, unidentified agent isolated from the intestinal tract of mice obtained a few years ago from commercial farms (3). This agent, which has been transmitted through several generations of SPF mice now produces in them depressions of the weight curve even more pronounced and lasting than those reported in the preceding paper of this series (3). As will be described in a later publication, the most active preparations of the agent so far obtained do not cause either paralysis or death of the infected mice, do not appreciably shorten their life span, but markedly decrease their body size.[1]

The present study was undertaken to define some of the metabolic changes caused in SPF mice by oral infection with the filterable agent(s). At various periods of time after infection, noninfected and infected SPF mice were com-

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[1] Attempts to identify the viral agent(s) responsible for growth depression have been hampered by the difficulty of obtaining consistent growth in tissue culture.

Antibody titrations in the serum of adult mice infected neonatally with the intestinal homogenate described in reference 3 and used in the present study have revealed no increase (measured either by hemagglutination inhibition or by complement fixation) for the following viruses: PVM, reovirus type 3, Theiler's encephalomyelitis (GD VII), K, polyoma, Sendai, mouse adenovirus. The serum of infected animals exhibited a very slight increase in complement fixation titer for mouse hepatitis virus, but the significance of this change is questionable.

Administration of terramycin or 1-methyl isatin 3-thiosemicarbazone before, during, and after infection did not affect in any way the growth curve of infected animals.

pared with regard to chemical composition of organs, changes in the nitrogen balance, and incorporation of ^{14}C-amino acids.

Materials and Methods

Infective Material.—The infective material used in these experiments had the following history. During October, 1967, 2-day old COBS mice (see below) were infected with a mouse embryo tissue culture of the agent reported in reference 3. 6 days after infection, the infected animals were sacrificed, their intestines collected aseptically, homogenized with a Teflon grinder, and the material passed through Millipore filters of 0.45 μ porosity. This filtrate was used to contaminate another group of 2-day old COBS mice which were sacrificed 6 days later. The intestines of these animals were collected and immediately frozen. They have been maintained ever since at −70°C. The frozen intestines have provided the infective material used in all experiments recorded in the present paper.

For each experiment, the frozen material was homogenized, 5 ml Tris-buffered salt solution being used per individual intestine. The homogenate was clarified through 0.45 μ Millipore filter. Two drops of this filtrate (approximately 0.05 ml) was administered per os to 2-day old COBS mice (3). The controls received the same volume of Tris-buffered salt solution.

Experimental Animals.—All experiments reported in this paper were carried out with SPF animals of the COBS colony (Caesarian-Obtained, Barrier-Sustained, Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Females were transferred to our laboratory during the 2nd wk of pregnancy; the date of birth of their young was recorded. Each litter was reduced to eight young.

All animals were maintained in Isocap plastic cages (Lab Cages Inc., Kennett Sq., Pa.) and given water and D&G pellets (Dietrich and Gambrill, Frederick, Md.) ad lib. They were weaned at 21 days of age.

Chemical Analyses of Organs.—Groups of 10 infected and 10 control males were sacrificed at 4 wks of age. The liver, kidneys, brain, and femur muscle were weighed and immediately processed or quick-frozen. The water contents, total lipids, and ash were determined by the AOAC method (4). DNA and RNA were determined by spectrophotometry. Protein was determined colorimetrically (5).

Nitrogen Balance Studies.—For this part of the study, six control and nine infected males, 4 wk old, were acclimatized in metabolic cages for 1 wk. Urine was collected daily, acidified, and kept under toluene. The cage washings were added to the sample and the pools of 4-day urine specimens made up to approximately 80 ml with distilled water. Total nitrogen was determined from two aliquots by the micro-Kjeldahl method.

Feces were collected daily and 4-day pools homogenized. Fecal nitrogen was determined from two aliquots by the same method as for urinary nitrogen determination.

The average nitrogen content of the D&G diet consumed was determined in three independent estimations. The amount of nitrogen retained was calculated as the difference between nitrogen intake and the combined urinary and fecal loss. The nitrogen balance study was continued for 16 days.

An attempt was made to determine the comparative effects of a biological stress on infected and control animals. To this end, both groups were administered the antituberculous vaccine BCG at the end of the 16-day period of nitrogen balance studies. Each animal received by the intravenous route 0.2 ml BCG (4×10^7 bacilli living units of the BCG strain produced by the Institut de Microbiologie et d'Hygiène, Université de Montréal). Following BCG administration, the animals were maintained for another 16 days. Changes in body weight, daily food intake, nitrogen excretion, and nitrogen retention were determined by the methods used in previous studies.

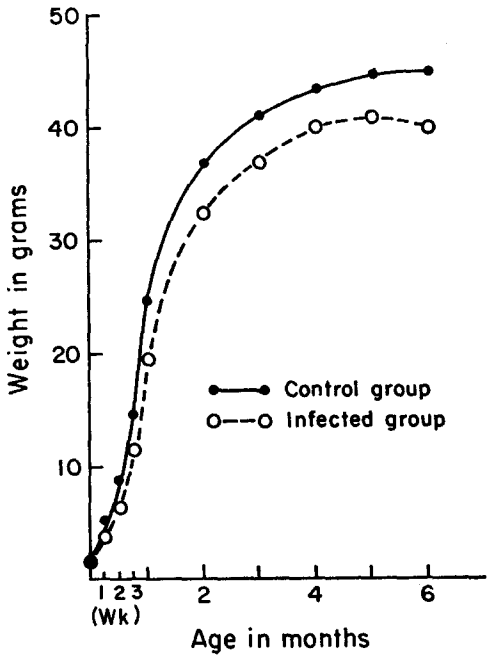

FIG. 1. Weight curves of male SPF mice infected neonatally with intestinal filtrate. Each point represents the average for 30 males at birth. Some animals were progressively removed from the experiment in the course of the study. There were still 12 control and 16 infected mice after 6 months.

Amino Acid Incorporation.—Groups of 10 infected and 10 control males, 4 wk of age, were used for this phase of the study. Each received 14 μc/100 g body weight of ^{14}C-amino acid mixture (uniformly labeled) (NEC-445 New England Nuclear Corp., Boston, Mass., specific activity, 40 mc/matom of carbon). The dose was administered by the intravenous route in a volume of 0.2 ml. The animals were sacrificed 24 hr after injection of the mixture. The liver, kidneys, brain, femur muscle, spleen, and thymus were then rapidly removed and homogenized separately in distilled water, using a glass homogenizer with a Teflon pestle. Portions of the homogenates were precipitated with 5% trichloroacetic acid in an ice bath for 60 min. The precipitates were clarified by centrifugation and washed with 95% ethanol saturated with sodium acetate, ethanol–ether mixture (3:1), and ether. The radioactivity was measured by the combustion method, absorbing the $^{14}CO_2$ combustion product in phenethylamine, and counting with a Packard liquid scintillation spectrometer, model 3003 (6).

Determination of Free Amino Nitrogen in Blood.—The free amino nitrogen in the blood was measured to determine the effect of the amino acid pool on amino acid incorporation in the various organs. To this end, 10 infected and 10 control males, 16 wk of age, were sacrificed and their blood collected. Red blood cells were precipitated from the plasma with 10% trichloroacetic acid and the amino nitrogen of the supernatant fluid was determined by the chelation with copper ion and titration of combined copper by iodometry (7).

TABLE I

Effect of Neonatal Infection on Body Weight of Male SPF Mice

Age	Controls		Infected	
	No. of animals	Body weight	No. of animals	Body weight
		g		g
Birth	30	1.48 ± 0.00*	30	1.47 ± 0.00
3 wk	30	14.6 ± 0.23	30	11.5 ± 0.17‡
6 months	12	45.4 ± 0.98	16	40.1 ± 0.71‡

* Mean of the respective group ± standard error of mean.
‡ $P < 0.01$.

TABLE II

Effect of Neonatal Infection On Organ Weights of Mice

	Controls*	Infected*
Body weight, g.....	24.9 ± 0.48	17.0 ± 0.95‡
Organ	Weight in g/100 g body wt	
Liver	7.74 ± 0.258§	8.20 ± 0.305
Kidneys	1.52 ± 0.030	1.57 ± 0.070
Brain	1.85 ± 0.058	2.36 ± 0.110‡
Heart	0.497 ± 0.016	0.541 ± 0.030
Lungs	0.786 ± 0.028	1.02 ± 0.073‡
Testes	0.490 ± 0.020	0.470 ± 0.026
Spleen	0.373 ± 0.026	0.471 ± 0.036‖
Thymus	0.523 ± 0.021	0.503 ± 0.021

* Average for 10 males in each group, 4 wk of age.
‡ $P < 0.01$.
§ Mean of the respective group ± standard error of mean.
‖ $P < 0.05$.

RESULTS

General Observations on the Infected Mice

Depression of Growth.—Comparative weight trends for control and infected male mice are shown in Fig. 1 and Table I. The difference in body weight could be detected as early as 5 days after infection and persisted from then on. When the body weights had leveled off at 6 months of age, the body weight of the infected males was 13.2% smaller than that of the control males.

Organ Weights.—The wet organ weights of male mice from the control and infected groups at 4 wk of age are shown in Table II. Both the total body

TABLE III

Effect of Neonatal Infection on Various Constituents of Mouse Organs

	Controls*				Infected*			
Body weight, g..	24.0 ± 0.48				17.0 ± 0.95‡			
Constituents	mg/g wet tissue				mg/g wet tissue			
	Liver	Kidney	Brain	Muscle	Liver	Kidney	Brain	Muscle
DNA	2.22 ± 0.12§	1.78 ± 0.11	1.06 ± 0.06	0.39 ± 0.02	2.03 ± 0.14	1.95 ± 0.11	1.12 ± 0.04	0.29 ± 0.02‖
RNA	14.0 ± 0.66	9.93 ± 0.46	4.72 ± 0.20	0.98 ± 0.07	15.6 ± 0.88	10.7 ± 0.34	4.41 ± 0.26	0.84 ± 0.05
Protein	230 ± 7.5	170 ± 3.4	118 ± 4.8	207 ± 3.2	214 ± 11.0	162 ± 3.4	102 ± 3.1‖	186 ± 5.5‡
Water	696 ± 3.9	752 ± 4.3	787 ± 4.9	753 ± 9.0	704 ± 3.1	763 ± 4.0	796 ± 3.5	754 ± 7.7
Ash	15.0 ± 0.5	14.9 ± 0.60	13.8 ± 0.6	12.4 ± 0.9	14.2 ± 0.5	14.9 ± 0.6	12.6 ± 0.4	13.3 ± 1.4
Total lipid	52.6 ± 2.5	46.7 ± 3.3	78.8 ± 1.9	62.8 ± 7.5	49.3 ± 2.0	41.6 ± 2.9	74.3 ± 1.6	62.4 ± 4.6

* Average for 10 males in each group, 4 wk of age.

‡ $P < 0.01$.

§ Mean of the respective group ± standard error of mean.

‖ $P < 0.05$.

weights and the actual wet weights of the individual organs were smaller in infected than in control animals. In the case of brains, lungs, and spleen, the ratio of organ weights to body weights was higher in the infected than in the control animals.

Chemical Determination on Various Organs.—As seen in Table III, there were no significant differences between the infected and control groups with regard to water, ash, RNA, and total lipids. Furthermore, no significant difference was noted in the DNA content of liver, kidney, brain, and in the protein content of

TABLE IV

Effect of Neonatal Viral Infection and of BCG Vaccination on Nitrogen Balance of Mice

	Controls*		Infected*	
	Before BCG	After BCG‡	Before BCG	After BCG
Body weight, g.....	26.4 ± 0.67§	32.5 ± 1.22	21.7 ± 0.30‖	28.6 ± 0.56‖
Daily food intake, g...	4.9 ± 0.2	4.1 ± 0.1	4.7 ± 0.0	4.1 ± 0.0
Nitrogen	mg N/kg body wt per day		mg N/kg body wt per day	
Intake	821 ± 15.8	591 ± 18.0	892 ± 9.0¶	670 ± 13.2¶
Urinary excretion	394 ± 18.7	267 ± 16.0	426 ± 14.2	321 ± 8.8‖
Fecal excretion	147 ± 8.0	114 ± 8.1	195 ± 6.0‖	170 ± 7.3‖
Retention	280 ± 19.3	210 ± 13.4	271 ± 14.6	179 ± 14.6

* Average for 6 control and 9 infected males, 4 wk of age.

‡ Intravenous injection of 0.2 ml BCG (4×10^7 viable bacillary units).

§ Mean of respective group ± standard error of mean.

‖ $P < 0.01$.

¶ $P < 0.05$.

liver and kidney. However, the infected group showed significantly lower values for muscle DNA and for brain and muscle protein.

Nitrogen Balance Studies.—The results of the nitrogen balance studies are summarized in Table IV. The mean daily food intake, expressed in terms of unit body weight was larger for the infected animals than for the controls.

Both groups were in positive nitrogen balance throughout the periods of study. There was no significant difference in urinary nitrogen excretion or in the nitrogen balance between them, but the infected animals excreted more fecal nitrogen.

The stress resulting from injection of the BCG vaccine brought about a marked decrease in food intake, urinary nitrogen, and fecal nitrogen of both

groups. Following BCG administration, however, the food intake of infected animals continued to be larger than that of the control group; furthermore, the infected animals excreted some 20% more nitrogen in their urine and some 49% more in their feces than did the controls. The nitrogen balance in the infected group was 14% lower than in the control group.

TABLE V

Effect of Neonatal Infection on Incorporation of ^{14}C-Amino Acid into Various Organs

	Controls*	Infected*
Body weight, g.....	24.9 ± 0.48‡	17.0 ± 0.95§
Organ	cpm/ mg protein	cpm/mg protein
Liver	487 ± 22.0	357 ± 23.4§
Kidney	477 ± 12.3	323 ± 27.0§
Muscle	143 ± 11.9	104 ± 9.4‖
Thymus	303 ± 25.7	232 ± 25.9
Spleen	190 ± 17.2	171 ± 19.3
Cerebellar cortex	211 ± 7.5	174 ± 14.9‖
Cerebral cortex	199 ± 7.5	141 ± 10.9‖
Brain white matter	212 ± 9.7	159 ± 13.6§

* Average for 10 males in each group, 4 wk of age.
‡ Mean of respective group ± standard error of mean.
§ $P < 0.01$
‖ $P < 0.05$.

TABLE VI

Blood Level of Free Amino Nitrogen

Milligrams Nitrogen/100 ml blood	
Control	Infected*
6.57 ± 0.35‡	5.60 ± 0.26§

* Average for 9 males in each group, 16 wk of age.
‡ Mean of respective group ± standard error of mean.
§ $P < 0.05$.

^{14}C-Amino Acid Incorporation and Free Amino Acid Pool.—The results of incorporation of ^{14}C-amino acid into the acid-precipitable fractions of various organs of control and infected mice are shown in Table V. In general, the ^{14}C-amino acid uptake in the liver and kidney was much higher than in the brain or muscle; incorporation was distributed evenly among the cerebral cortex, the cerebellar cortex, and the brain white matter.

The ability to incorporate ^{14}C–amino acid in the liver, kidney, brain, and muscle was significantly lower in the infected animals than in the control animals, but no significant difference could be recognized between the two groups in the thymus and spleen.

As seen in Table VI, the free amino acid pool, expressed by the free amino nitrogen in the blood, was significanty lower in the infected group than in the control group.

DISCUSSION

The lasting impairment of growth caused by neonatal administration to SPF mice of the viral agent discussed in this and the preceding paper (3) is associated with metabolic disturbances that persist long after the initial infectious event. Not only do the infected animals remain unusually small throughout their life span; they also continuously exhibit an abnormal ratio between the weights of their lungs, spleen, and brain, and their total body weight. The concentration of DNA in muscles, and of protein in brain and muscle is also lower in infected than in control animals.

The fact that infected mice consumed more food per unit of body weight than did normal animals rules out the possibility that persistence of growth depression was due to loss of appetite.

There was no significant difference in urinary nitrogen and in nitrogen balance between the two groups; but the fecal nitrogen excretion was higher in infected animals. Since the difference between nitrogen intake and fecal output was essentially the same in infected and control animals, the continued depression of growth of the former group can hardly be attributed to impairment of digestion or absorption.

When BCG vaccine was administered to mice 6 wk after neonatal infection with the virus, the physiological stress thus produced determined in the infected mice a larger wastage of nitrogen through the urine and the feces than was observed in the control animals. The nitrogen retained was 14.3% smaller in the infected than in the control group.

The decreased ability of infected mice to incorporate ^{14}C–amino acid into the acid precipitable fraction of their various organs constitutes further evidence for some abnormality in their protein metabolism. Impairment of incorporation was observed in the liver, kidney, muscle, brain, but not in the thymus and spleen.

The free amino acid pool, as represented by the blood level of free amino nitrogen, was lower in infected than in control animals. Since this fact excludes the possibility that reduced ability to incorporate ^{14}C–amino acid was due to the large size of the free amino acid pool, it appears probable that depression of body weight can be attributed to abnormal protein metabolism or utilization.

A hypothesis worth considering is that the lasting metabolic disturbance and depression of growth caused by neonatal infection may be the result of a decrease in the numbers of cells produced in certain organs. The test for this hypothesis is based on the fact that the DNA content of diploid nuclei appears to be constant in a given species. The total amount of DNA found in an organ would therefore constitute an indication of the number of diploid cells this organ contains (8).

It has been reported that malnutrition during early life causes a decrease in weight of various organs, as well as a decrease in the DNA, RNA, and protein they contain (9). Malnutrition appears to cause a reduction in cell numbers without changing cell size. In the present study, however, decrease in DNA was observed only in muscle. This fact suggests that decrease in the numbers of muscle cells may be one of the fundamental mechanisms for impairment of growth of infected mice.

SUMMARY

A lasting depression of body weight was consistently produced in SPF mice by infecting them orally 2 days after birth with a nonlethal, bacteria-free filtrate, prepared from the intestine of young SPF mice previously infected with an unidentified agent.

Neonatal infection caused a decrease of muscle DNA and of muscle and brain protein in the adults. No other effect was detected in the chemical composition of various organs.

Incorporation of ^{14}C–amino acid into the acid precipitable fractions of liver, kidney, muscle, and brain was lower in infected than in control animals. No difference in incorporation was recognized in the thymus and spleen.

The free amino acid pool of adults, measured as blood levels of free amino nitrogen, was decreased by neonatal infection.

Surprisingly, the food intake of young animals infected neonatally was higher than that of the controls, measured on the basis of body weight. Their fecal excretion of nitrogen was also higher.

The comparative responses of infected and control adults to a stressful situation was measured by giving them intravenously the antituberculous vaccine BCG. Under these conditions, the mice infected neonatally excreted some 20% more nitrogen in their urine and 40% more in their feces than did the controls.

The mechanisms through which neonatal infection caused a lasting weight depression are discussed in the light of these metabolic findings.

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