DEPRESSION OF ANAEROBIC GLYCOLYSIS OF EMBRYONIC TISSUE BY WESTERN STRAIN OF EQUINE ENCEPHALOMYELITIS VIRUS. PREVENTION OF THIS EFFECT BY SPECIFIC IMMUNE SERUM

By JOSEPH VICTOR, M.D., and C. H. HUANG, M.D.

(From the Research Service, First Division, Goldwater Memorial Hospital, Department of Hospitals, City of New York, and the Department of Medicine, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, October 14, 1943)

Titration and neutralization of the Western strain of equine encephalomyelitis virus (W.E.E.) by means of tissue cultures have been demonstrated by Huang (2, 3). Employing the technique devised by Simms and Sanders (1) he found that minced skeletal muscle of chick embryo, incubated with W.E.E. for 48 hours, failed to grow when transferred to plasma. Examination of the plasma patches for growth of the tissue was made 48 hours after the latter was planted in the plasma. When immune anti-W.E.E. horse serum was mixed with virus and incubated for 48 hours with embryonic skeletal muscle, the growth inhibiting effect of the virus on the tissue was prevented.

It seemed probable that embryonic tissue with impaired ability to grow might have a disturbance in cellular function. Many studies have demonstrated that growing tissues, both embryonic and tumor, have higher glycolytic rates than resting tissues of the same cell type. If embryonic tissues infected with W.E.E. had a lower glycolytic rate than normal tissue, the demonstration of such a disturbed function would simplify and shorten the time necessary for carrying out titration and neutralization tests. Recent studies of Racker and Kabat (4) indicated that the brain and spinal cord of mice infected with the Lansing strain of poliomyelitis virus have a lower rate of anaerobic glycolysis than normal, although the respiratory rate is unaltered.

The following experiments are concerned with the effects of the Western strain of equine encephalomyelitis virus on the anaerobic glycolysis of embryonic chick tissue. It was found that a decrease in anaerobic glycolysis occurred in infected embryonic tissue and that it could be prevented by the action of specific immune serum.

Methods

Embryonic tissue was incubated with W.E.E. virus propagated by either tissue cultures or mouse brain passage. One drop of W.E.E. virus was inoculated into test tubes containing 1 cc. of serum ultrafiltrate and salt solution and 10 pieces of either minced whole embryo, embryo from which brain and spinal cord had been removed, or

embryonic skeletal muscle. The type of tissue was varied to learn which might be most suitable for metabolic studies. It was found that the results were not significantly different with any of the tissue mixtures. The mixtures of virus and tissue were incubated at 37.5° for various periods as indicated below. At appropriate times, aliquots of the mixed tissue were removed for growth studies on a plasma patch and for measurements of anaerobic glycolysis in a respirometer previously described (5). In each determination the tissue weighed 1 to 3 mg. moist weight and usually 1 to 4 fragments were employed. The respirometers contained a modified bicarbonate-Ringer-glucose solution which consisted of 100 parts 0.154 M NaCl, 3 parts 0.154 M $CaCl_2$, 4 parts 0.154 M KCl, 0.025 M $NaHCO_3$, and 0.012 M glucose. Determinations of anaerobic glycolysis (anaerobic acid production in the presence of glucose) were conducted in 95 per cent N_2 and 5 per cent CO_2 at 37.5°C. for 1 to 2 hours.

RESULTS

The Effect of the Western Strain of Equine Encephalomyelitis Virus on the Rate of Anaerobic Glycolysis of Chick Embryo Tissues

In Table I are recorded the individual rates of anaerobic glycolysis of normal and infected embryonic tissues. The results are tabulated according to the type of embryonic tissue employed, as whole embryo, embryo from which brain and spinal cord had been removed, or embryonic skeletal muscle. Comparisons between normal and infected tissues derived from the same source are listed as well as the percentage difference between them. The interval between the addition of the virus to the tissue suspension and the metabolism measurement is also indicated.

There was a marked decrease in the rate of anaerobic glycolysis of the tissue mixed with virus as compared with its normal control. The decrease appeared slightly greater in the skeletal muscle than in the other tissues. This decrease was apparent 2 to 4 days after the infection in each of 20 comparisons, the percentage difference varying from 18 to 78. However, tissues examined at intervals of 4 hours or 6 days after addition of virus showed no depression of anaerobic glycolysis as compared with normal controls. Indeed, after an interval of 6 days, there was a fall in the rate of anaerobic glycolysis of normal tissues and a slight elevation in that of infected tissues so that the rates of both were approximately the same.

Simultaneous observations on growth and metabolism made at various intervals after mixing virus and tissue confirm previous findings in showing inhibition of growth at intervals between 4 hours and 4 days. However, in the present experiments growth inhibition was occasionally partial instead of complete as had been found before (2, 3). This slight difference in degree of growth inhibition caused by W.E.E. virus may have been due to loss of virulence. In spite of this the alterations in anaerobic glycolysis of the infected

tissue are unequivocal. No explanation for the "recovery" of the tissues from the influence of the virus after 6 days is available. Since growth inhibi-

TABLE I

Comparison of Rates of Anaerobic Glycolysis of Normal Chick Embryo Tissue and That Exposed to Western Strain of Equine Encephalomyelitis Virus

Duration of exposure	Type of tissue	Anaerobic glycolysis: Normal	Anaerobic glycolysis: Tissue and virus	Difference
days		*c.mm. CO_2/gm. moist weight/min.*		*per cent*
2	Whole embryo	6.7	3.3	−51
2		7.1	4.3	−40
2		7.2	2.0	−72
4		2.9	0.8	−72
4		5.8	2.5	−57
Mean		5.9	2.6	−56
2	Embryo without brain or spinal cord	3.1	1.1	−65
2		5.1	3.9	−24
2		6.6	5.4	−18
4		10.2	6.0	−40
4		10.1	4.4	−56
Mean		7.0	4.2	−40
4 hrs.	Embryonic skeletal muscle	8.7	9.4	+7
days		9.4	8.1	−14
2		6.9	3.4	−51
		3.9	2.4	−38
		8.3	4.2	−49
		10.3	2.6	−75
3		8.0	2.1	−74
		5.3	2.4	−55
4		9.5	2.5	−74
		8.2	1.8	−78
			2.0	
			2.4	
		6.6	3.6	−45
		6.4	2.1	−67
6		3.5	4.6	+31
		3.5	3.8	+9
		4.5	4.3	−5

tion by the W.E.E. virus was partial in many instances it is possible that after 6 days the tissue may have completely "recovered" from the effects of the virus. Further studies are necessary to clarify this point.

The Effect of Anti-W.E.E. Immune Serum on the Inhibition of Anaerobic Glycolysis of Embryonic Tissue Caused by W.E.E. Serum

Equal volumes of dilutions of virus were mixed with immune anti-W.E.E. horse serum prepared by the Lederle Laboratories, Inc., New York. Antimeningococcus horse serum served as control. The serum virus mixtures were incubated at 37.5°C. for 30 minutes. Then 1 drop of each mixture was added to about 15 pieces of embryonic skeletal muscle in each tube which contained 1.5 cc. of serum ultrafiltrate. The mixture of virus and serum was incubated with the tissue at 37.5°C. for 48 hours.

TABLE II

Comparison of Anaerobic Glycolytic Rates of Chick Embryo Muscle Mixed with W.E.E. Virus and W.E.E. Antiserum and That Mixed with W.E.E. Virus and Antimeningococcus Serum

Virus concentration	Sera		Difference
	Anti-W.E.E.	Antimeningococcus	
	Anaerobic glycolysis		
	c.mm. CO_2/gm. moist weight/min.		*per cent*
10^{-1}	7.0	2.6	−63
10^{-1}	7.9	6.6	−17
10^{-1}	10.6	2.9	−73
10^{-1}	2.2	0.4	−82
10^{-1}	6.6	1.3	−80
10^{-3}	7.6	4.1	−42
10^{-4}	8.2	5.0	−39
10^{-4}	9.8	2.3	−76
10^{-4}	8.2	5.1	−38
10^{-4}	4.2	6.2	+47
Mean.....	7.23	3.85	−47

An interval of 2 days was selected for the incubation period because it was sufficient to demonstrate inhibition of anaerobic glycolysis in chick embryonic tissue by W.E.E. virus. At this time aliquots of the tissue were examined for growth by transfer to plasma and studied 48 hours later. The rest of the tissue was studied in the respirometer.

In Table II are recorded the results of 10 experiments in which the rate of anaerobic glycolysis of chick embryo skeletal muscle exposed to W.E.E. virus and anti-W.E.E. serum was compared with similar tissue exposed to W.E.E. virus and antimeningococcus serum. Nine of these 10 comparisons show that anti-W.E.E. immune serum prevented depression of anaerobic glycolysis of embryonic skeletal muscle caused by W.E.E. virus. The exceptional instance in which no depression was observed occurred in tissue which, through mishap, was incubated at 37.5°C. for only 31 instead of 48 hours. For 17 hours it was

kept at room temperature of 25°C. This tissue was exposed to a relatively dilute dose of virus, namely 10^{-4} of W.E.E. virus.

The mean rate of anaerobic glycolysis of the tissues exposed to W.E.E. virus and antiserum, Table II, was equivalent to 7.23 c.mm. CO_2 per gm. moist weight per minute while that for similar normal tissues without virus and antiserum was 7.35 c.mm. CO_2 per gm. moist weight per minute. Comparison of Table II, skeletal muscle at 2 day interval with W.E.E. virus and antiserum, Table I normal untreated skeletal muscle, 2 day interval, reveals these data. In other words there was no significant difference in the rate of anaerobic glycolysis of normal embryonic skeletal muscle and that exposed simultaneously to W.E.E. virus and antiserum.

In contrast to these results obtained with W.E.E. antiserum and W.E.E. virus are those observed with antimeningococcus serum and W.E.E. virus. The mean rate of anaerobic glycolysis of tissue exposed to W.E.E. virus and antimeningococcus serum was equivalent to 3.85 c.mm. CO_2 per gm. moist weight per minute while that of similar tissue and W.E.E. virus without antimeningococcus serum was 3.15 c.mm. CO_2 per gm. moist weight per minute. These data are derived from Table II, virus and antimeningococcus serum at 2 day interval as compared with Table I, skeletal muscle and virus, 2 day interval. The mean difference in rates of anaerobic glycolysis between the tissues exposed to virus either with or without antimeningococcus serum is not significant. Protection against this metabolic effect of W.E.E. virus on embryonic tissue is afforded by specific anti-W.E.E. serum and not by antimeningococcus serum.

DISCUSSION

The experiments described above show that W.E.E. virus inhibited the anaerobic production of acid (anaerobic glycolysis) by embryonic chick tissue. Inhibition of anaerobic acid production was demonstrable 2 days after exposure of the tissue to the virus and persisted for as long as 2 days thereafter. This depressed function of embryonic tissue caused by W.E.E. virus was not confined to special tissues of the embryo because it occurred in the whole embryo, in embryo from which brain and cord has been removed, and also in embryonic skeletal muscle. Nor is this effect specific for the chick embryo *in vitro* or for the W.E.E. virus since Racker and Kabat (4) have observed similar metabolic alterations in brain and spinal cord of mice infected with poliomyelitis virus. Depression of anaerobic acid production of tissues may be part of the mechanism whereby viruses produce tissue death. Further studies on the enzyme systems concerned in this altered function are desirable.

Demonstration of the depressed anaerobic production of acid by tissue exposed to W.E.E. virus requires less time than that required for observing either altered growth in tissue culture or the pathogenic effect in animals.

Furthermore the technique does not require any of the procedures employed with tissue culture. Adequate evidence of a decreased acid production is obtained in an hour after the previously incubated tissue is placed in the respirometer, while the other methods mentioned above require several days.

Since specific immune serum, *i.e.* anti-W.E.E. serum, protects the tissue against depression of its anaerobic acid production caused by W.E.E. virus, this technique could probably be employed as a method for titration and neutralization of the virus. Obviously similar studies on other viruses are indicated.

The experiments outlined above have one drawback, which is essentially a practical one, in applying them to general laboratory procedure. The technique for operating respirometers of the design employed in these experiments requires considerable experience. For this reason a much simpler method (6) has been developed for observing the changes in acid production in tissues caused by W.E.E. virus. It can be applied to other viruses. The method depends upon demonstrating a difference in the rate of anaerobic production of acid between normal embryonic tissues and those exposed to virus. The production of acid can be observed by noting a change in color of an indicator dye in unbuffered isotonic salt solutions. From data obtained thus far, it appears that the rate of anaerobic acid production of normal tissues is $1\frac{1}{2}$ to 5 times as great as that of similar tissue exposed to W.E.E. virus for 2 days. The average rate of anaerobic acid production of normal tissue is equivalent to about 7 c.mm. CO_2 per gm. moist weight per minute while that of similar tissue exposed to W.E.E. virus is about 3 c.mm. CO_2 per gm. moist weight per minute. If normal tissue produces enough acid to change the color of the dye indicator in which it is immersed in 1 hour then the tissue exposed to W.E.E. virus would require $2\frac{1}{3}$ times as long or $2\frac{1}{3}$ hours. By employing suitable quantities of tissue and substrate, as little as 1 tissue fragment can be used for such a test. Experiments based on the above principles have been carried out with W.E.E. and other viruses and have been reported elsewhere (6).

SUMMARY AND CONCLUSIONS

Studies were made on the effect of mixing the Western strain of equine encephalomyelitis virus (W.E.E.) and embryonic tissue on the rate of anaerobic glycolysis of the tissue. Whole chick embryo, chick embryo from which brain and spinal cord had been removed, and embryonic skeletal muscle were employed.

1. W.E.E. virus depressed the rate of anaerobic glycolysis of embryonic tissues within 2 days after its addition to the tissue. The decrease in anaerobic glycolysis varied from 17 to 82 per cent and was apparent 2 to 4 days after

the addition of the virus. No significant effect of the virus was observed 4 hours and 6 days after mixing it with the tissue.

2. Anti-W.E.E. immune serum prevented the inhibiting action of W.E.E. virus on the anaerobic glycolysis of embryonic skeletal muscle.

BIBLIOGRAPHY

1. Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, **33,** 619.
2. Huang, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51,** 396.
3. Huang, C. H., *J. Exp. Med.*, 1943, **78,** 111.
4. Racker, E., and Kabat, H., *J. Exp. Med.*, 1942, **76,** 579.
5. Victor, J., *Am. J. Physiol.*, 1935, **111,** 477.
6. Huang, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54,** 160.