9. Keiser, H. R., Henkin, R. I., and Kare, M. R., Proc. Soc. Exptl. Biol. Med. **129**, 516 (1968).

10. Crawhall, J. C., Scowen, E. F., and Watts, R. W. E., Brit. Med. J. **2**, 588 (1963).

11. McCall, J. T., Goldstein, N. P., Randall, R. V., and Gross, J. B., Am. J. Med. Sci. **254**, 13 (1967).

Received Dec. 26, 1968. P.S.E.B.M., 1969, Vol. 131.

Gastric Mucosal Antibodies and Iron Absorption* (33926)

M. J. MURRAY AND NELL STEIN

Department of Medicine, University of Minnesota, Minneapolis, Minnesota 55455

Circulating antibodies to parietal cells have been found in the serum of patients with iron deficiency anemia, especially if such patients have achlorhydria. Dagg *et al.* (1) reported 11 positive antibody tests in 33 patients with iron deficiency anemia and histamine fast achlorhydria, but only two in 31 iron-deficient patients with acid. The observations of Shearman *et al.* (2) were similar. They found 7 positive tests in 8 anemic patients with total achlorhydria. None of these 7 had circulating antibodies to intrinsic factor. With these observations in mind we decided to explore the possibility that antiparietal cell antibodies might inhibit iron absorption. Since we had no human clinical material available, we chose the following experimental model in rats: we studied the effect of (i) antigastric mucosal antibody on iron absorption by normal rats and rats made anemic by iron depletion, (ii) antigastric mucosal antibody on the secretion of hydrochloric acid by rats in the unlikely event that depression of acid secretion would decrease iron absorption, and (iii) antigastric mucosal antibody on the binding of iron by gastric juice *in vitro*.

Methods. Preparation of antigastric mucosal antibody (AGMS). This was prepared by a modification of the technique of Walder and Lundseth (3) for the production of antiparietal cell antibody. Mucosa was scraped from the stomachs (except the antral region) of 110 male Sprague-Dawley rats in five batches. The tissues were minced, rinsed, and the cells were extracted after incubation with collagenase. The material was then centrifuged at 5° for 15 min at 800 rpm and washed with buffer several times. The deposits of each batch were pooled and disrupted with ultrasound at 38 kcps for 3 min and then resuspended. After centrifugation at 5° for 1 hr at 1000 rpm the supernatant was collected; and had a protein content of 7 mg/ml by the Folin-Ciocalteau method. Four New Zealand white rabbits were immunized as follows with this material. Each received four injections, once weekly, of 5 mg of protein made up to a volume of 2 ml with 30% aluminum hydroxide. The serum was harvested 1 week later from the rabbits and the presence of antigastric mucosal antibodies was demonstrated by reacting the serum against the original antigen on Ouchterlony double gel diffusion plates. Control antigens were prepared from pooled rat spleen, kidney, and serum using the technique of Gold and Freedman (4). The control antigens were adjusted to a protein concentration of 10 mg/ml and reacted against antigastric mucosal serum on Ouchterlony plates. Reactions were noted with all antigens but many more lines developed with gastric antigen than with the others.

Preparation of gastric juice for binding studies. Gastric juice was collected from 20 normal and 20 anemic mature male Sprague-Dawley rats by the technique of Shay *et al.* (5). Each specimen was measured and filtered through cheesecloth. The pH was determined, the specimen was titrated to pH 10–11 for 30 min with 10% potassium hydroxide and then back titrated to pH 7 with

* Supported by the John Hartford Foundation and by the National Institute of Arthritis and Metabolic Diseases Research Grant No. AM 10976-01.

TABLE I. Mixtures Tested.

Contents (ml) Tube no.:	1	2	3	4	5	6	7	8	9
Saline	0.5	0.5	0.5	0.5					1.0
Normal rat gastric juice (NRGJ)	0.5				0.5	0.5			
Anemic rat gastric juice (AnRGJ)		0.5					0.5	0.5	
Normal rabbit serum (NRbS)			0.5		0.5		0.5		
Antirat gastric mucosa rabbit serum (AGMS)				0.5		0.5		0.5	
^{59}Fe as ferrous citrate	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
BSA[a] coated activated charcoal	10	10	10	10	10	10	10	10	10

[a] BSA = bovine serum albumin.

0.1 N sulfuric acid to inactivate pepsin. Specimens were stored at —20°. The anemia was produced by feeding the rats on a low iron diet and repeated bleedings from the tail. The mean hemoglobin in gram/100 ml of this group at the time gastric juice was collected was 8.9. The normal animals were fed on the same diet with a supplement of 275 ppm of iron as ferrous ammonium sulfate. Their mean hemoglobin concentration (g/100 ml) was 14.0.

Expt. I. Effect of antigastric mucosal antibody on iron absorption. Using methods we have already described (6), the absorption of ^{59}Fe was determined in four groups of 10 anemic rats (mean hemoglobin ± SEM = 10.2 ± 0.50). Each group received 0.25 μCi of ^{59}Fe as ferrous citrate without carrier but Group I got 0.5 ml of normal rat gastric juice (NRGJ) plus 0.5 ml of normal rabbit serum (NRbS); Group II got 0.5 ml of NRGJ plus 0.5 ml of AGMS; Group III got 0.5 ml of anemic rat gastric juice (AnRGJ) plus 0.5 ml of NRbS; and Group IV got 0.5 ml of AnRGJ plus 0.5 ml of AGMS. Percentage absorption was determined, 12 days after dosing, in a Packard scintillation detector no. 446.

Expt. II. The effect of anti-rat gastric mucosa rabbit serum (AGMS) on the Secretion of Gastric Acid. A 0.5-ml dose of antigastric mucosa serum was injected daily for 1 week into the tail veins of 12 mature male Sprague-Dawley rats. They were then fasted for 48 hr and a 4-hr collection of gastric juice was made by the technique of Shay *et al.* (5). The results were compared with those obtained in 12 untreated rats.

Expt. III. The effect of antigastric mucosal serum (AGMS) on the in vitro *binding of ^{59}Fe by gastric juice.* In our preliminary studies, activated charcoal coated with bovine serum albumin absorbed ferrous iron as ferrous citrate but not ferrous iron attached to large protein molecules, e.g., rabbit serum. Using a modification of the techniques devised by Ardeman *et al.* (7), and Gottleib *et al.* (8), we studied the mixtures recorded in Table I. The methods and materials used were as follows: glassware, tubes, and pipettes were kept iron free by washing them in 5 *N* nitric acid; 0.1% EDTA; and repeatedly rinsing them in de-ionized, distilled water. Merck 18351 activated charcoal was coated with bovine serum albumin, Kohn Fraction V-I in the following proportions: de-ionized, distilled water, 100 ml; charcoal, 2.5 g; albumin 0.5 g. Radioferrous citrate solution (Abbott) was diluted with de-ionized, distilled water to provide 14,000 to 15,000 cpm of radioactivity per 0.1 ml.

Final mixtures were made in 14-ml capped "autoclear" plastic centrifuge tubes. These mixtures consisted of gastric juice plus saline or test sera mixed for 10 sec. The radioferrous citrate was added and mixed for 10 sec

TABLE II. Percentage Absorption by Anemic Rats of 0.25 μCi of ^{59}Fe as Ferrous Citrate Mixed with Combinations of Gastric Juice, Normal Rabbit Serum, and Antigastric Mucosal Antiserum.

^{59}Fe plus	Absorption (%) ± SEM
NRGJ + NRbS	24.58 ± 1.86
NRGJ + AGMS	16.10 ± 1.23
AnRGJ + NRbS	75.12 ± 5.29
AnRGJ + AGMS	59.03 ± 4.71

and then the whole was incubated at 37° for 30 min in a water bath. Ten ml of prepared charcoal were added and mixed well. The tubes were centrifuged at high speed for 15 min. The supernatant was removed by disposable pipette and the radioactivity was determined in it and the charcoal pellet.

Results. Expt. I. The mean values ± SEM for absorption of ^{59}Fe combined with mixtures of NRbS, AGMS, NRGJ, and AnRGJ by anemic rats are recorded in Table II. It is clear that when antigastric mucosal antibody is present in rabbit serum it significantly reduces absorption of ^{59}Fe; $p = 0.01$ in both situations.

Expt. II. No significant differences in pH, volume, total acid, and acid per hour in milliequivalents were noted between the two groups. A variety of techniques of administration, sites of administration, and doses of AGMS were also tried with similar results.

Expt. III. The results are recorded in Table III. With this method using neutralized and depepsinized gastric juice the following observations could be made: (i) normal rat gastric juice slightly binds ^{59}Fe; (ii) anemic rat gastric juice has virtually no iron binding capacity; (iii) normal rabbit serum has considerable iron binding capacity, but this is strikingly reduced by mixing it with either normal or anaemic rat gastric juice; and (iv) Antigastric mucosal serum also binds ^{59}Fe well, but this binding is not reduced by mixing with either normal or anemic gastric juices.

Discussion. Antigastric mucosal serum clearly reduces the absorption of iron by rats and this phenomenon does not appear to be related in any way to the effect of antigastric mucosal serum on gastric acidity. Our *in vitro* studies suggest that the effect might be due to the formation of large molecules of iron antibody antigen complex which are poorly absorbed.

When we examine the *in vitro* studies closely we find that gastric juice reduces the binding of iron by normal rabbit serum. How can we account for this? We would like to suggest that a small molecule is present in gastric juice which preferentially binds iron, making it less available for binding to the large protein molecules in rabbit serum. Our data also suggest that when iron is bound to the complex which results from mixing gastric juice and antigastric mucosal serum, its absorption in the animals, normal and anemic, is significantly reduced. We believe that this is most likely due to the inability of the animal to absorb iron molecules of this size.

These ideas can be incorporated into a theory to explain the association of iron-deficiency anemia, achlorhydria, and circulating parietal cell antibodies. Gastric mucosal injury may lead to the production of anti-parietal cell antibody which combines as an antibody–antigen reaction with the small molecular iron compounds (which facilitate absorption of iron normally) found in gastric juice. In cases with adequate acid and pepsin, these molecules are broken down, thus, allowing near normal absorption of iron to take place. Without acid, however, these large complexes might neither be broken down nor absorbed. The end result is further iron depletion.

TABLE III. Binding of ^{59}Fe as Ferrous Citrate to Activated Charcoal Coated with Bovine Serum Albumin.[a]

Mixture	On charcoal	In supernate
Saline	100	0
NRGJ	77.93	22.07
AnRGJ	86.93	13.07
NRbS	14.61	85.39
AGMS	14.85	85.15
NRGJ + NRbS	61.91	38.09
NRGJ + AGMS	21.50	78.50
AnRGJ + NRbS	53.82	46.18
AnRGJ + AGMS	15.70	84.30

[a] See mixtures in Table I.

Summary and Conclusions. Antigastric mucosal antibody reduces iron absorption in normal and anemic rats. This appears to result from the formation of large antibody–antigen complexes with the iron which are not easily absorbed. Such a mechanism might account for the idiopathic hypochromic microcytic anemia associated with complete achlorhydria and circulating parietal antibody. In this situation lack of acid might prevent degradation of the large molecules to a more easily absorbed form. A simple technique for studying iron binding *in vitro* was devised.

1. Dagg, J. H., Goldberg, A., Anderson, J. R., Beck, J. S., and Gray, K. G., Brit. Med. Jr. **1**, 1349 (1964).

2. Shearman, D. J. C., Delamore, I. W., and Gardner, D. C., Lancet **1**, 845 (1966).

3. Walder, A. and Lundseth, J., Proc. Soc. Exptl. Biol. Med. **112**, 494 (1963).

4. Gold, P. and Freedman, S. O., J. Exptl. Med. **121**, 439 (1965).

5. Shay, H., Sun, D., and Gruenstein, M., Gastroenterology **26**, 906 (1964).

6. Murray, M. J. and Stein, N., Gastroenterology **53**, 38 (1967).

7. Ardeman, S. and Chanarin, I., Brit. J. Haematol. **11**, 305 (1965).

8. Gottleid, C., Lau, K., Wasserman, L. R., and Herbert, V., Blood **25**, 875 (1965).

Received Jan. 16, 1969. P.S.E.B.M., 1969, Vol. 131.

Effect of Neuraminidase on the Accumulation of Alpha-Aminoisobutyric Acid in HeLa Cells* (33927)

D. M. Brown[1] and A. F. Michael
(Introduced by R. L. Vernier)

Department of Pediatrics and Laboratory Medicine, University of Minnesota Medical School, Minneapolis, Minnesota 55455

Sialic acid residues are major constituents of the surface of plasma membranes and contribute to the negative change of the surface membranes (1, 2). Several functional alterations of cells may be induced by removal of sialic acid from the surface membrane. These include enhanced phagocytosis (2, 3), inhibition of viral (4) or mycoplasma (5) induced hemagglutination, unmasking of cell surface antigens (6), inhibition of cell aggregation (7), and increased cell deformability (8).

The present studies illustrate how membrane composition can selectively be changed and related to discrete functional interrelationships of cells with each other and their environment. This method can similarly be utilized to study the effect of altered plasma membrane composition as applied to the function of the membrane as a barrier to the passage of simple chemicals. The effect of removal of surface membrane sialic acid from HeLa cells on the transport of alpha-aminoisobutyric acid was studied.

Materials and Methods. HeLa cells in monolayer culture (plaque bottle) were incubated in Eagle's basic media with Hanks' BSS (10% fetal calf serum) and used during the logarithmic phase of growth. The growth media were removed and the monolayer was washed twice with Hanks' BSS (pH 7.4). Four ml of Hanks' BSS containing 0.1–0.2 μCi ^{14}C-1-alpha-aminoisobutyric (^{14}C-AIB) acid (New England Nuclear Corp., sp act 5.5 mCi/mmole) were added and the cells were incubated at 37° for 30 or 60 min. Incubation media were quickly removed and the cells washed three times with cold BSS. The cells were suspended in 2.0 ml of 0.02% Na_2EDTA. An aliquot was taken for determination of protein (9) and the remainder

* Supported by the National Institutes of Health Grant No. 12357, the Graduate School of the University of Minnesota and the American Heart Association.

[1] Career Development Awardee of the USPHS.