QUANTITATIVE IMMUNOCHEMICAL STUDIES WITH THE PURIFIED FACTOR IN MOUSE MILK CONNECTED WITH MAMMARY CARCINOMA*

By MICHAEL HEIDELBERGER, Ph.D., SAMUEL GRAFF, Ph.D., and C. D. HAAGENSEN, M.D.

With the Technical Assistance of Arnold Powell and Anton Samuel

(From the Department of Medicine and the Institute for Cancer Research, College of Physicians and Surgeons, Columbia University; and the Presbyterian and Francis Delafield Hospitals, New York)

(Received for publication, November 9, 1951)

Although one characteristic of a virus is its possession of immunological specificities different from those of its host (1) the finding of a new specificity in the tissues of a diseased animal may reflect changes brought about by the virus, and so not indicate the virus itself (2). The distinctive specificity of a virus is, moreover, not necessarily absolute. Frequent instances of the occurrence of the specificities of normal tissue in virus preparations have been noted and some of these have been studied by quantitative immunochemical techniques (3, 4). The capacity for neutralization by specific antibodies is also characteristic of viruses, in common with other biologically active antigens.

Because of its immunological and biological characteristics the factor in mouse milk connected with mammary carcinoma has been considered to be a virus. The factor was shown to stimulate the production of neutralizing antibodies (5, 6) and, on the basis of parallel experiments with antisera to tissue extracts containing the factor and with antisera to normal mouse tissues it was stated (6) that the "agent is antigenically different from mouse tissue and not derived therefrom." This has also been indicated by qualitative precipitin tests (7). The present investigation was undertaken (*a*) because of continuing uncertainties as to the viral nature of the factor, (*b*) because quantitative immunochemical data exist for relatively few viruses which are pathogenic for animals

* The work reported in this communication was carried out in part under the Harkness Research Fund of the Presbyterian Hospital, and in part under grants from the Rockefeller Foundation, Public Health Service, Ligget and Waters Fund, a fund given in memory of Katherine Converse Strong, and the Damon Runyon Memorial Fund of the American Cancer Society.

Read at the 4th Annual Scientific meeting of the Detroit Institute of Cancer Research, October 22–24, 1951. The writers wish to acknowledge helpful comments from Dr. Thomas Francis, Jr., and Dr. Samuel Albert in the ensuing discussion.

(3, 4), and (*c*) because of the great importance of immunological specificity in the study of the nature of incitants to cancer.

Materials and Methods

Milk Factor.—Highly purified preparations of the mouse milk factor (hereinafter designated as F) have been reported by several groups of investigators (8, 9). The material used in the present studies was prepared from Paris R III strain milk according to the method of Graff *et al.* (8).

Mouse milk was also obtained from normal animals of the C57 strain. After centrifugation at low speed in the cold and removal of much fat (fraction C) the skim milk was centrifuged for 1 hour at 10,000 R.P.M. in the chilled rotor of a Servall centrifuge. The white sediment was designated fraction A and the clear milk serum fraction B. Fraction A was washed once with saline containing 0.02 to 0.05 M $CaCl_2$ and 1:10,000 merthiolate and was then taken up in the same solvent. At least two different proteins, separable with the help of Na_2SO_4, were present in fraction B, but ordinarily the mixture was used both for the injection of rabbits and for the absorption of sera.

Extract of Normal Mouse Breast Tissue.—Normal C57 non-lactating mice were exsanguinated and breast tissue was excised from 28 animals by Dr. H. T. Randall and allowed to stand in a CO_2 refrigerator overnight. After addition of 10 ml. of cold saline and 10 ml. of 1.25 per cent $NaHCO_3$ solution the thawed tissue was rapidly comminuted in the cold in a Waring blendor with a small metal mixing chamber. Low speed centrifugation removed fat and large particles. The turbid pink solution was run through a folded filter several times in the cold and was finally allowed to drip into toluene. The mixture was centrifuged in the cold and the aqueous layer again filtered, remaining opalescent. 1 per cent by volume of 1 per cent merthiolate[1] solution was added.

Immunization of Rabbits.—After a preliminary bleeding designated by the subnumeral 0, rabbits MF 1 and 3 were each given 7 intravenous injections totalling 10 mg. of F in the course of 9 days and were bled 6 days later (bleedings 1_1 and 3_1). 4 weeks later a similar course of 10 injections totalling 17 mg. F was given and the rabbits were bled 6 days later (bleedings 1_2 and 3_2).

A suspension of F containing 22 mg. in 2.5 ml. was homogenized in a mortar with 2.5 ml. of aquaphor and 5 ml. of paraffin oil containing 6.25 mg. of dry tubercle bacilli (10). Three 1 ml. portions of the mixture were injected intramuscularly at weekly intervals into rabbits MF 7, 9, and 10. Bleedings were taken 10 days, 1 month, 2 months, and in the case of Nos. 9 and 10, 3½ months, after the third injection. In this series each rabbit received a total of 7 mg. of F.

Similarly, 4 ml. each of C57 milk fractions A and B, containing 96 and 56 mg. of protein, respectively, were mixed with 9.4 mg. of dry tubercle bacilli, 4 ml. of aquaphor, and 8 ml. of paraffin oil. Two rabbits, MM 1 and 2, were given three intramuscular injections of approximately 1 ml. of mixture A at weekly intervals and two rabbits, MM 3 and 4, were given similar injections of mixture B. The amounts of protein injected were approximately 18 and 10 mg., respectively. Bleedings were taken 12 days, 1 month, and 1½ months after the last injection.

Analyses of Antisera.—Purified lots of F were taken up in a small volume of saline and diluted to contain about 100 to 150 μg. N per 0.2 ml. Merthiolate was added to a final concentration of 1:10,000. Usually 0.20 ml. of the solution was added in duplicate to 0.50 or 1.0 ml. samples of anti-F serum diluted with 1 ml. of saline in tapered pyrex centrifuge tubes of

[1] Manufactured by Eli Lilly and Co., Indianapolis.

7 to 8 ml. capacity (11). A serum blank was also run, but F blanks were discontinued when it was found that negligible quantities of N remained in the tubes after the usual centrifugations and washings according to (11, 12). Most of the anti-F sera precipitated rapidly upon mixing with F. In such instances the number of absorptions necessary for precipitation of all of the antibody could be reduced by adding a second portion of F before allowing the mixtures to stand 48 hours in the cold. After centrifugation the supernatants were transferred quantitatively to a new set of tubes and the analyses were repeated, numerous absorptions of a serum occasionally being found necessary. If, after centrifugation, the supernatants and washings were entirely clear and thus showed no evidence of uncombined F, the entire amount of F nitrogen added was subtracted from the total N found by the micro-Kjeldahl or Markham (13) method. The former procedure was preferred for precipitates containing more than

TABLE I

Successive Absorptions of Rabbit Antiserum to Mouse Milk Factor with Homologous Antigen

Serum No.	Vol. antiserum dilution	Amount antigen nitrogen	Total N precipitated	Antibody N precipitated	Ratio antibody N: antigen N in precipitate	Appearance of supernatants or washings
	ml.	*μg.*	*μg.*	*μg.*		
Pool 7_1, 9_1 absorbed	2.5	101	246	145	1.4	Clear
with C57 milk fraction B (Table II)		51	106	55	1.1	"
		303	558	255	0.8	"
		101	158*	57	0.6	"
		101	117	29‡		Slightly opalescent
		101	81	20‡		Opalescent
7_1, 9_1 unabsorbed	0.5	101	205	104	1.0	Clear
		101	113	34§		
		51‖	18	5‡		

* One determination lost.

‡ Total N ÷ 4, on basis of decreasing antibody:antigen ratios.

§ Total N ÷ 3, on basis of previous ratio

‖ Supernatants combined and 101 μg. antigen N added.

150 μg. N. Usually after the first or second precipitation the supernatants and/or washings showed a slight turbidity. Although this was possibly due to antigen-antibody complexes, for simplicity the turbidity was assumed to be caused by excess F. The composition of any precipitate remaining in the tubes after washing was then calculated as in the examples given in Table I, under the assumption of a regular diminution in the values of the antibody N: antigen N ratios found in the preceding absorptions of the serum. While the necessity of calculating the amount of antibody precipitated in the final absorptions introduces an uncertainty into the total values found, it is believed that the method used ensures figures that are too low rather than too high.

Addition of amounts of F solution varying between 0.05 and 0.2 ml. resulted in the precipitation of minimal quantities of N from normal rabbit sera or from antisera to the fractions of C57 mouse milk.

Quantitative analyses of antisera with fraction A of C57 milk gave uncertain results, partly because this fraction required prolonged centrifugation at high speed and partly because its solubility in sera increased with increasing pH. The data obtained even after neutralization of

TABLE II

Precipitation by Fractions of C57 Mouse Milk (A, B) and by Milk Factor (F) in Antisera to Mouse Milk Factor

Data calculated to 1.0 ml. undiluted serum.

Rabbit serum Nos.	Antigen	Antibody N precipitated	Second antigen added	Antibody N precipitated	Third antigen added	Antibody N precipitated	Fourth antigen added	Antibody N precipitated
		μg.		μg.		μg.		μg.
$MF1_1$	110 F	89^6 77^4						
1_2	110 F	119^6	B	21*				
	A		B	131*	111 F	126^4		
3_1	110 F	176^6						
	A		B^4	39*	NBT‡	5*	111 F	116^4
3_2	110 F	279^7	B	20*				
	113 B	97^4*	A^4		NBT	0	111 F	223^3
	Ct§	1*						
Normal pool	A	15‖	B^5	*ca* 2*	110 F	5		
" " 7_0, 9_0	B	>2*						
7_1, 9_1	A^3		B^3	14*	109 F	264^4		
	B	21*	109 F	263^6				
	110 F	294^3						
7_2	B^3	72*	A	0^3	111 F	¶		
	111 F	494^7						
7_3	112 F	328^6; after inactivation at 56°C., 262						
9_2	A^3	35‖	B^6	39*	110 F	95^{2}**		
					NBT	7*		
					B	1*	110 F	125^3
	110 F	135^4						
9_3	112 F	168^4						
9_4	112 F	80^4						
10_0	111 F	2						
10_1	111 F	$112,^3$ 106^5						
	B	7^4*	A		111 F	100^3		
10_2	111 F	$94,^3$ 95^4						
10_3	111 F	$90,^3$ 66^3						
10_4	111 F	$28,^3$ 22^3						

Superscripts denote number of absorptions.

* Total N.

‡ Extract of C57 mouse breast tissue.

§ Crystalline chymotrypsin, 4.4 μg. N.

‖ Run in triplicate, suspension of A washed three times.

¶Unstable solution of A used; precipitated during absorption of serum with F.

** After another absorption with A and 3 more with B, 83 μg. of antibody N were precipitated by 111 F. The serum had by this time been diluted seven-fold.

several sera are therefore omitted. An actual solution of fraction A, made from a suspension with a little $NaHCO_3$, was scarcely more helpful. Although the solution gave a small specific precipitate with an antiserum to fraction A and yielded no appreciable precipitate with anti-F, a considerable proportion of the protein precipitated on standing, so that quantitative data on residual antibodies in the anti-F serum and on absorptions of other sera were invalidated. Anti-F sera were, however, absorbed several times with suspensions of fraction A, as noted in Table II, in order to remove, if possible, any antibodies to this fraction. It has recently been found that phosphate solutions of fraction A are more stable than those prepared with bicarbonate.

Since fraction B was known to be heterogeneous, values of total N precipitated are given for analyses with this material. In most instances far less N was precipitated than was added, particularly in the final absorptions of anti-F sera in which several milliliters of 1 to 2 per cent solutions of fraction B were added.

Chymotrypsin and Antisera to Chymotrypsin.—Since chymotrypsin (Ct) was used for the digestion of mouse milk proteins in the preparation of F (8) it was considered advisable to determine whether or not any new specificity shown by F might be due to Ct. With the help of adjuvants (10) as above a precipitating antiserum was obtained in a rabbit. This serum gave no precipitate with F, nor did Ct precipitate appreciable quantities of N from an anti-F serum (Table II).

DISCUSSION

The experiments described in the preceding section and summarized in the tables show clearly that the mouse milk factor (F) isolated from Paris R III strain milk according to the method of Graff *et al.* (8), is a potent antigen. Precipitating antisera were readily obtained with as little as 10 to 17 mg. of F when this was injected intravenously in multiple doses, and with 7 mg. when F was mixed with aquaphor and tubercle bacilli (10) and divided into three portions which were given intramuscularly at weekly intervals. With the latter procedure peak antibody levels, in one instance as much as 0.5 mg. of antibody nitrogen per ml., were attained in from 10 to 30 days and fell off quite rapidly, possibly on account of the small quantity of antigen used.

Presumably because of a particle weight estimated roughly at 300×10^6, and possibly also because the preparations were not entirely pure (8), relatively large quantities of F were required to absorb the antibody from the stronger anti-F sera. Under the assumption that all of the F nitrogen added was precipitated as long as antibody remained and the supernatants and washings were clear, the antibody N:antigen N ratios in the precipitates were low and often less than 1, reversing the usual trend with smaller antigens (Table I).

Further details of the reaction between F and homologous antibody will be given in another communication, since the main object of the present study was to determine whether or not F possessed a specificity distinct from those of the proteins of mouse milk or was different from the proteins of mammary tissue from which milk is secreted.

For this purpose the fat was skimmed from the milk of normal C57 mice, which does not contain F, and the proteins were roughly divided into two fractions, one, A, centrifugable in the Servall centrifuge at 10,000 R.P.M. and obviously consisting in large measure of casein, the

other, B, the remaining proteins in the supernatant milk serum. Antisera in rabbits were obtained to these two fractions, although the anti-A sera were very weak. Anti-F sera were also exhaustively absorbed with suspensions of A, and with solutions of B in increasing volume until no further evidence of precipitation could be obtained.

The amounts of precipitate formed in the anti-F sera and the numbers of absorptions are given in Table II. Unfortunately, accurate analytical data could rarely be obtained with A, even when the sera were brought to pH 6.7–6.8 and the suspensions of A were sufficiently coarse to sediment readily at 2,000 to 3,000 R.P.M. in the No. 2 International refrigerated centrifuge. In no instance was agglutination of A particles observed, nor, in the one case in which a soluble preparation of A was used after absorption with B, was any specific precipitate formed in the best of the antisera. Unfortunately, estimation of residual anti-F was vitiated in this instance by the instability of the solution of A, which began to precipitate during subsequent analyses both in the presence and absence of this serum and others. In general, the anti-F sera gave more precipitate with B than did normal rabbit sera, but the nitrogen precipitated appeared to come only in part from the anti-F content. This was shown both by the high proportion of anti-F remaining even after numerous absorptions with B, and by the appreciable precipitates obtained with B after several sera were first precipitated exhaustively with F (for example, sera 1_2 and 3_2, Table II). The extract of C57 mouse breast tissue, in amounts of 0.2 to 1.5 ml., containing 116 to 870 μg. of N, respectively, failed to yield appreciable precipitates in anti-F sera, nor did the presence of this material, which must have contained serum proteins as well as tissue proteins, inhibit precipitation of most of the anti-F present in the original serum.

It is also evident that the specificity of F is unrelated to that of the chymotrypsin used in its preparation, as the enzyme gave practically no precipitate in serum 3_2, containing 279 μg. of anti-F N per ml. (Table II) and F precipitated only 2 μg. N from an antiserum to chymotrypsin which yielded a total of 190 μg. of precipitable N with 31 μg. of enzyme N.

As noted in Table III, precipitation of F in anti-B sera is very slight, so that there is no marked cross-reaction in this direction. Since there was little antibody in the anti-A sera attempts are now being made to obtain better sera.

While the data assembled in the present study supply additional evidence that the mouse milk factor shows an immunological specificity different from those of normal mouse proteins (6, 7) the evidence is not yet conclusive. The necessity for the use of another strain of mice as a source of factor-free milk and tissue proteins raises the question as to whether or not a closer relationship would have been found had it been possible to use material from the homologous strain. Not only must extracts of other mouse tissues and organs be tested against antisera to F, but before one can assert with assurance that the F particle does not contain the specificity of any protein present even in

C57 milk in appreciable amount it will be necessary to obtain more stable solutions of the milk fraction A and stronger antisera to this fraction. However, the indications thus far point to very little cross-reactivity between this fraction and F. The cross-reactivity between F and fraction B of C57 mouse

TABLE III

Reactions of C57 Mouse Milk Fractions and Mouse Milk Factor in Antisera to Fractions of C57 Mouse Milk

Calculated to 1.0 ml. undiluted serum.

Rabbit serum Nos.	Antigen	Antibody N precipitated	Second antigen added	Total N precipitated	Third antigen added	Total N precipitated
		μg.		μg.		μg.
Antisera to fraction A						
NM 1_0	110 F	4^2	B	+	A	?
			NBT	0		
1_1	110 F	13^2	B	13*	A	?
1_2	F	4^2				
	A	21*				
	B	9^{4*}				
2_0	F	0				
	B	0				
	A	?				
2_2	F	2^2				
	B	30^{4*}				
	A	?				
Antisera to fraction B						
3_0	F	0				
	B	0				
3_1	F	4^3				
	B	81^{5*}				
3_2	B	96^{4*}				
4_0	F	0				
	B	0				
4_1	F	8^3				
	B	44^{5*}				
4_2	B	38^{4*}				

* Total N.

milk is more extensive although not all of the precipitate would seem due to antibody to F, as explained above. In some anti-F sera 5 to 10 mg. of B sufficed for complete absorption with this fraction, but in others up to 5 to 6 volumes of 1 to 2 per cent solutions were necessary. In all cases, however, more than one-half of the original anti-F was still precipitable in the supernatant, and often nearly as much as in the unabsorbed sera. It is evident, therefore, that F differs from any protein present in fraction B in appreciable amount. F is, moreover,

unlike influenza virus in this respect, for the preparations of influenza particles studied quantitatively (3, 4) appeared to contain a specificity of normal chick embryo tissue and completely exhausted antisera to chick embryo. The embryo did not, however, exhaust anti-viral sera.

Work along the above lines is being continued and other studies on the immunochemical relationships of F such as the neutralization of F by known quantities of antibody N, are being carried out.

SUMMARY

Antisera to the mouse milk factor (F), containing up to 0.5 mg. of antibody nitrogen per ml. are readily obtainable in rabbits, especially when adjuvants are added to the antigen.

Quantitative data on the anti-F content of the sera are given, also on the total nitrogen precipitated by the non-casein fraction of C57 mouse milk which does not contain F.

The antisera were also absorbed with casein fractions but the difficulties in the way of quantitative analysis were not overcome.

Antisera to fractions of C57 mouse milk were also obtained and the reactions of F and the C57 proteins with these sera were studied.

The results, as far as they go, are in accord with the earlier belief that the milk factor possesses an immunological specificity not present in normal mouse tissues.

BIBLIOGRAPHY

1. Reviewed *in* Viral and Rickettsial Infections of Man (T. M. Rivers, editor), J. B. Lippincott, Philadelphia, 1949.
2. Hughes, T. P., *J. Immunol.*, 1933, **25,** 275.
3. Kabat, E. A., *J. Immunol.*, 1943, **47,** 513. Knight, C. A., *J. Exp. Med.*, 1944, **80,** 83; 1946, **83,** 281.
4. Cohen, S. S., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57,** 358.
5. Andervont, H. B., and Bryan, W. R., *J. Nat. Cancer Inst.*, 1944, **5,** 143.
6. Green, R. G., Moosey, M. M., and Bittner, J. J. *Proc. Soc. Exp. Biol. and Med.*, 1946, **61,** 115.
7. Imagawa, D. T., Green, R. G., and Halvorson, H. O., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68,** 162.
8. Graff, S., Moore, D. H., Stanley, W. M., Randall, H. T., and Haagensen, C. D., 4th International Cancer Research Conference, Abstracts, 1947, 144; *Cancer*, 1949, **2,** 755.
9. Passey, R. D., Dmochowski, L., Astbury, W. T., and Reed, R., *Nature*, 1947, **160,** 565; (with Johnson, P.,) **161,** 759; **165,** 107; Passey, R. D., Dmochowski, L., Reed, R., and Astbury, W. T., *Biochim. et Biophysic. Acta*, 1950, **4,** 391.
10. Freund, J., Hanson, K. J., Hough, H. P., Sommer, H. E., and Pisani, P. M., *J. Immunol.*, 1948, **60,** 383.
11. Heidelberger, M., and MacPherson, C. F. C., *Science*, 1943, **97,** 405; **98,** 63.
12. Heidelberger, M., and DiLapi, M. M., *J. Immunol.*, 1949, **61,** 153.
13. Markham, R., *Biochem. J.*, 1942, **36,** 790.