ANTIBODY-MEDIATED ACTIVATION OF A DEFECTIVE β-D-GALACTOSIDASE*

II. Immunological Relationship Between the Normal and the Defective Enzyme*

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Enzymes have often been used for immunological studies as antigens (1–4), and as reagents to tag either antigens (5, 6) or antibodies (7, 8), because of the ease, sensitivity, and specificity of enzyme assays. Moreover, antibodies may affect the catalytic properties of enzymes, thereby providing a convenient tool to measure the interaction of the two molecules. The most frequently observed type of interaction leads to inactivation of the enzyme (9, 10); however, it has been recently reported that antibodies can cause an increase in the enzymatic activity. The activation is usually not more than 3- or 4-fold, with the exceptions of a 70-fold increase shown for the penicillinase of a *Bacillus subtilis* mutant (11) and a 500-fold increase for a genetically defective β-D-galactosidase from *Escherichia coli* (12).

The latter system seemed of interest to us, since, in addition to the large activation factor, β-D-galactosidase is a well-studied enzyme offering two main advantages over other enzymes. Namely, it can be assayed at the single molecule level (13) and its synthesis can be genetically controlled so as to obtain a series of polypeptides which are useful for the immunological characterization of the enzyme (14), since they range from relatively small polypeptides to molecules differing from the normal enzyme by a single amino acid (15, 16).

In the first paper of this series (12) we described the purification and bio-

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chemical characterization of a defective β-D-galactosidase (AMEF)[1] which is activable by specific antibodies directed against the normal enzyme (Z). The AMEF was extracted from a point mutant of *E. coli* shown to have a defect in the structural gene for β-galactosidase (17). The sedimentation rate of AMEF is comparable to that of the tetramer form of β-galactosidase. This, together with studies of specificity of induction, indicated that AMEF is the product of an altered Z gene, probably differing from the wild-type enzyme by one amino acid. The major difference in the physical properties of AMEF and Z is that the former is more electronegatively charged as shown by electrophoresis in acrylamide gel (unpublished results).

The reaction of AMEF with the antibody is characterized by an increase in enzymatic activity directly proportional to the concentration of either antibody (12) or Fab fragments (18). The results of kinetics studies are consistent with the postulate that activation of AMEF results from a single hit involving a discrete number of antigenic determinants (18). Further evidence that one molecule of Fab can activate one molecule of AMEF has been obtained by direct measurements of single molecules of the AMEF-Fab complex.[2] In addition, the latter studies have shown that, with some antisera, the activity of the AMEF-Fab complex can reach a maximum value of about one-third of that of a normal Z molecule.

Given the properties of the AMEF-antibody system as summarized above, it follows that it could serve well as a prototype to study the protein-to-protein interactions leading to conformational and functional changes, reactions for which there is increasing interest among biologists. From the immunological point of view, the binding of an activating antibody molecule to the corresponding antigenic site of AMEF can be compared with the specific reaction of an anti-hapten immunoglobin and the protein-bound hapten. Accordingly, this system could be used to test the general validity of the concepts derived from experimentation with carrier-hapten models by extending them to given determinants of a native protein. As a first step in this direction, the immunogenic and antigenic properties of AMEF and Z are described in this article.

Materials and Methods

Diluents.—Buffer B contained 10 mM tris (hydroxymethyl) aminomethane (Tris), 10 mM $MgCl_2$, 0.1 M NaCl, and 0.05 M 2-mercaptoethanol and its pH was adjusted to 7.05 (23°C) with acetic acid. The complete buffer was prepared daily by adding the mercaptoethanol to

[1] *Abbreviations used in this paper:* AMEF, a genetically defective β-D-galactosidase; EU, enzyme unit, the amount of β-D-galactosidase which hydrolizes 10^{-9} mole of substrate/min; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; P.U., the amount of serum necessary to precipitate 1 EU of Z, taking 50% precipitation as the end point; *s*, galactosidase activity after centrifugation; *t*, galactosidase activity before centrifugation; Z, normal β-D-galactosidase of *E. coli*.

[2] Rotman, B. Unpublished observations.

the stock salt solution. Whenever the buffer was used without mercaptoethanol, it is noted in the text.

Chemicals.—Isopropyl-β-D-1-thiogalactopyranoside and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Mann Research Labs. Inc., New York, Tris was obtained from Sigma Chemical Co., St. Louis, Mo., and sodium lactate was from Fisher Scientific Company, Pittsburgh, Pa.

Bacterial Strains and Conditions of Culture.—A β-D-galactosidase constitutive mutant of *E. coli* (3·300) grown in tryptone-lactate broth was used for the production of normal β-D-galactosidase (19). For AMEF, either W6101 (F^-, *lac*Z 201) or S 167 (F^-, *lac*I, *lac*Z 201) was used. The former is an inducible strain, while the latter, a recombinant between W6101 and S 164 (HfrC, *lac*I), synthesizes AMEF constitutively (Ellis and Rotman, unpublished results). The bacteria were grown under aeration at 37°C in minimal medium (20) containing 0.4% sodium lactate as sole carbon source. For induction, 5×10^{-4} M isopropyl-β-D-1-thiogalactopyranoside was added to the growth medium.

Assay of β-D-Galactosidase.—The enzymatic assay, identical for Z and AMEF, was done at 37°C in a 2 ml volume of buffer B containing 3 mM ONPG. The reaction was stopped by adding either 3 ml of 0.2 M Na_2CO_3 or 0.5 ml of 1.2 M Na_2CO_3 and its optical density at 420 mμ was measured and converted to *o*-nitrophenol concentration using a molar extinction coefficient (1 cm pathway) of 4700. An enzyme unit (EU) is defined as the amount of β-D-galactosidase which hydrolizes 10^{-9} mole of substrate/min under the above conditions.

Purification of Z and AMEF.[3]—The procedures followed for the extraction of 3300, S167, and W6101 were similar. Unless noted otherwise, all operations were done between 0° and 4°C. The bacteria were harvested at a density of about 10^9 cells/ml, washed three times with buffer B without mercaptoethanol, resuspended in the same buffer to a density of about 10^{12} cells/ml, and then disrupted in a French pressure cell [a MSA sonicator set at 1.4 amp for 4 min]. The resulting extract was freed of particulate matter by centrifuging first at 27,000 *g* for 15 min in a Sorvall centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) and then at 97,000 *g* for 60 min in a Spinco model L ultracentrifuge (Spinco Div. Beckman Instruments Inc., Palo Alto, Calif.). The supernatant was mixed with ⅓ of the volume of saturated ammonium sulphate (70.6 g dissolved in 100 ml of water and adjusted to pH 7.2 with 5 M NaOH) and the precipitate formed was removed by centrifugation at 27,000 *g* for 10 min. The supernatant was again mixed with saturated ammonium sulphate until 33% [38%] saturation was reached. The mixture was allowed to stand for 20 min at 0°C [1 hr at 4°C] and the precipitate obtained was spun down at 27,000 *g* for 10 min, resuspended in a volume of 40% saturated ammonium sulphate equal to about three times that of the pellet, and stored at 4°C. Before experimental use, samples of the latter suspension were spun down and the pellet, after carefully removing the supernatant by a second brief centrifugation, was dissolved in buffer B and dialyzed for 24 hr against the same buffer [passed through a Sephadex G-25 column (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) (1.5 × 25 cm)] in order to eliminate the ammonium sulphate. For some experiments where AMEF was used at such high dilution that the salt concentration became negligible, the dialysis step was omitted.

The activity of β-D-galactosidase solutions prepared by this procedure was stable for several weeks at 4°C, while the activity of AMEF preparations decayed considerably under the same conditions. Therefore, the latter was prepared daily as described above from stock suspensions under 40% ammonium sulphate. The protein concentrations of AMEF and Z solutions were standardized by measuring their optical density at 280 mμ. For conversion to milligrams of protein per milliliter, the optical density was multiplied by 0.5.

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[3] Variations in procedures used in the Stockholm laboratory are indicated in square brackets.

Quantitative Assay of Immune Precipitation.—A given amount of Z, usually between 500 and 1000 EU, in a volume of 0.3 ml, was added to each of a series of tubes containing 0.2 ml of progressive dilutions of the antiserum to be tested. The tubes were incubated 30 min at 37°C [30 min at 37°C, then 60 min at 4°C], then centrifuged at 20,000 *g* for 10 min in a Clay Adams "micro-chemistry" centrifuge (Clay-Adams, Inc., Parsippany, N.Y.) [at 48,000 *g* for 20 min in a Sorvall RC2B]. The supernatant of each of the tubes after centrifugation was assayed for β-D-galactosidase activity. Taking the activity before and after centrifugation as t and s, respectively, the fraction precipitated is expressed as $1 - s/t$. The titer of a serum is determined in slight antigen excess by taking the tube with highest dilution of antiserum, in which more than 50% of the Z is precipitated, as the end point. Under these conditions the number of enzyme units precipitated by 1 μl of undiluted serum is calculated by multiplying

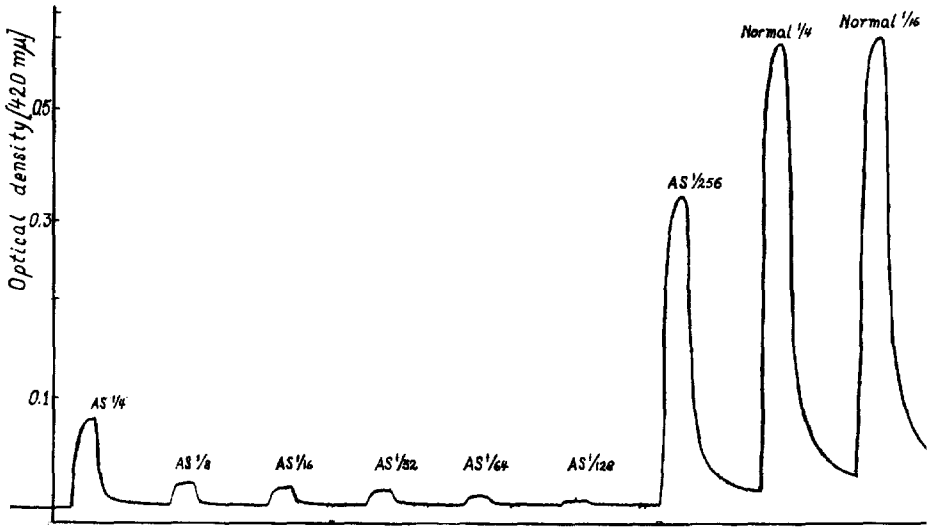

FIG. 1. Precipitation curve of Z and anti-Z made in a Technicon continuous analyzer. See Materials and Methods for details.

the original number of enzyme units present in the tube by the fraction precipitated and by the serum dilution factor, and then dividing the result by 200 (the microliters of serum in the tube).

For experiments involving a considerable number of β-D-galactosidase assays, a Technicon continuous analyzer was used (Technicon Co., Inc., Tarrytown, N.Y.). The supernatants obtained before and after precipitation of the antibody-enzyme complex were placed in the analyzer and assayed at a rate of 20/hr. The instrument was run with the same solutions used in the manual method, except for the ONPG which was 10^{-3} M. A titration of an anti-Z serum in the Technicon is shown in Fig. 1. From the graph, one can evaluate both the titer of the antiserum and the profile of precipitation showing that the zones of antibody excess, equivalence, and antigen excess are well delineated.

Assay of Activation of AMEF.—The activating capacity of antisera was measured by incubating 0.1 [0.05] ml of a given dilution of the serum with 0.1 [0.05] ml of a solution of AMEF containing about 100 μg protein/ml. After 30 min at 37°C, a 0.1 [0.02] ml sample of the mixture was assayed for β-D-galactosidase. The activity of the tube containing the highest

dilution of serum and showing a significant difference above a control with normal serum and AMEF was used for the calculation of the titer. For this, the increase in enzyme units elicited by 1 ml of diluted serum was multiplied by its dilution factor and the result was divided by 1000 to obtain the activation capacity of 1 μl of serum.

Animals and Immunization Procedures.—Male and female rabbits weighing between 2 and 5 kg each were used. Immunization was done by injecting subcutaneously into the subscapular muscle 1 ml of either Z or AMEF solution containing from 3 to 20 mg protein emulsified with 1 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). The same procedure was repeated for secondary stimulation, 1 month after the primary. At varying intervals beginning 3 wk after primary stimulation and 1 wk after challenge bleedings were done by heart puncture or by cannulating the central artery of the ear. The collected serum was kept at 4°C after sterilization by filtration through Millipore (Millipore Corp., Bedford, Mass.) [at −30°C in small samples].

Male mice of the A. Sn strain, bred at the Department of Tumor Biology, were utilized at the age of 6-8 months, both for immunization experiments and as recipients in transfer experiments. The standard immunization dose of Z and AMEF was 100 μg/mouse, emulsified in Freund's adjuvant (Difco). A total volume of 0.2 ml of the emulsion was injected subcutaneously into the abdominal region. To elicit a secondary response, the same procedure was repeated 1 month after the primary stimulation. Bleeding of the injected mice was done weekly by puncturing the retro-orbital venous sinus and the sera obtained were stored individually at −30°C until used.

Adoptive Transfer Procedures.—Donor mice were killed by decapitation 2–3 months after immunization and their spleens were teased in ice-cold Eagle's tissue culture medium by using two miniature toothed forceps. The resulting suspension was passed several times through a syringe mounted with an 18-gauge needle and then filtered through a stainless steel cloth of 200 mesh/in. to eliminate cell clumps, washed, and adjusted to a concentration of 10^8 cells/ml. A 0.2 ml sample of this suspension was given intravenously to each of the preirradiated recipient mice. Antigen challenge was given by injecting 0.2 ml of a solution of either Z or AMEF in buffer B containing 10 μg of protein. The recipients, usually eight per experimental group, were bled weekly after transfer. The irradiation conditions were as follows. A total dose of 600 R was administered with a Philips machine (Philips Electronic Instrument, Mount Vernon, N.Y.) set at 200 kvp, 15 ma. The inherent filter was 1 mm Al, the added filter, 0.5 mm Cu. The animals were placed 50 cm from the source, in a circular plexiglas container. A Philips integrating dosimeter was used; the dose rate was about 90 R/min.

RESULTS

Difference Between Anti-Z and Anti-AMEF Sera.—In contrast to Z, AMEF did not elicit formation of antibodies capable of activating AMEF, although the production of precipiting antibodies was similar to that of Z (Table I). This effect was observed in both rabbits and mice. By the criterion of the Ouchterlony technique, the cross-reaction between the anti-Z and anti-AMEF sera with regard to the two antigens is complete since no spurs were detected.

Failure of Z to Elicit Antibodies in Animals Presensitized with AMEF.—As shown in Table II, one injection of AMEF in Freund's adjuvant altered the immune response of mice to subsequent inoculation of Z so that no activiting antibodies were produced. This suppressing effect of AMEF was restricted to activating antibodies as shown by the fact that a normal secondary response

was observed with regard to precipitating antibodies. Identical results were obtained in rabbits, which were shown to remain incapable of responding with activating antibodies for prolonged periods of time (up to 2 yr) after a single injection of 1–5 mg of AMEF in Freund's adjuvant.

TABLE I

Precipitating and Activating Capacity of Anti-Z and Anti-AMEF Sera from Rabbits and Mice

	Titer	
	Precipitation*	Activation‡
Anti-Z (rabbit)	1250	69500
Anti-Z (mouse)	330	4750
Anti-AMEF (rabbit)	312	0§
Anti-AMEF (mouse)	300	0§
Normal serum‖	0	0

Given amounts of either AMEF or normal β-D-galactosidase were mixed with the indicated serum in order to test for activation or precipitation respectively according to procedure described in Materials and Methods.

* EU of Z precipitated at equivalence by 1 μl serum.

‡ Increase in EU produced by 1 ml serum in AMEF excess.

§ Not different from normal serum.

‖ Normal serum did not precipitate detectable amounts of enzyme. The activity of AMEF was not significantly enhanced.

TABLE II

Suppression of Activating Antibody Response by Sensitization with AMEF

Group	Immunization (day 0)	Titer (day 18)		Challenge (day 18)	Titer (day 34)	
	Dose	Activ.	Precip.	Dose	Activ.	Precip.
1	Freund's adjuvant	0.4	n.t.	none	2	0
2	Freund's adjuvant	n.t.	n.t.	12 μg Z	129	128
3	15 μg AMEF	0	32	12 μg Z	0	512
4	12 μg Z	99*	n.t.	12 μg Z	137	512

Mice were preimmunized according to the above schedule and tested for production of precipitating and activating antibodies. The titers are expressed in the same units used in Table I. Groups were composed of three animals, except for No. 3 which had six mice. All antigens were injected intraperitoneally in Freund's adjuvant. Activ., activating; Precip., precipitating; n.t., not tested.

*This group was tested on the 12th day.

Comparison Between the Immunogenicity of AMEF and Z Studied by Adoptive Transfer.—The suppressive effect of preimmunization with AMEF was subjected to a more stringent test by studying it during adoptive transfer, thus reducing the presence of preformed circulating antibodies or cell-bound antibodies. Suspensions of normal spleen cells, AMEF-immunized spleen cells, and

a 1:1 mixture of the two were each transferred to irradiated syngeneic mice. Immediately after the transfer, the animals were divided into two groups and each of them was challenged with an injection of 10 μg of Z/mouse, in either soluble form or mixed with Freund's adjuvant. This type of challenge was chosen in order to discriminate between a primary and a secondary response, since several cases are known in which small doses of soluble protein antigen fail to elicit a primary adoptive response in the mouse while effectively stimulating a secondary response (21, 22). After the transfer, the recipient mice were bled at weekly intervals and the sera were titrated for activating and precipi-

TABLE III

Comparison Between Adoptive Response of AMEF-Sensitized and Naive Spleen Cells

Group	Pre-treatment of donors	No. of cells transferred ($\times 10^7$)	Challenge with Z	Titer							
				day 7		day 14		day 22		day 28	
				Act.	Prec.	Act.	Prec.	Act.	Prec.	Act.	Prec.
1	AMEF	3	Soluble	0	0	0	0	150	36	140	76
2	AMEF	3	Freund's	0	20	0	40	264	200	153	290
3	AMEF 1.5 / None 1.5	mix	Soluble	0	0	0	0	52	16	32	20
4	AMEF 1.5 / None 1.5	mix	Freund's	0	5	0	20	104	70	96	145
5	None	3	Soluble	0	0	0	0	0	0	0	5
6	None	3	Freund's	0	0	0	0	121	28	310	70

The indicated numbers of spleen cells from either AMEF-primed donors, normal donors, or a mixture of the two types, were transferred to groups of X-irradiated recipients and challenged with 100 μg Z in soluble form or in Freund's adjuvant. Activating (Act.) and precipitating (Prec.) titers were determined in the serum at weekly intervals and are expressed in the same units used in Table I.

tating antibodies. The results of this experiment (Table III) show that the transfer of cells from animals immunized with AMEF, in contrast to normal cells, produced a secondary response with respect to both activating and precipitating antibodies after challenge with soluble Z (compare groups 1 and 5 of Table III) and that the response was proportional to the number of the sensitized cells injected (see group 3). The difference between the challenge with soluble Z and with Z in Freund's adjuvant indicates that, similar to other proteins, β-D-galactosidase in the soluble form and in the dose range tested fails to elicit a primary response in an adoptive transfer system.

The susceptibility of cells from mice preimmunized with Z to the challenge by AMEF in an adoptive transfer was also examined. The results (Table IV) show that AMEF can elicit a secondary response for both activating and pre-

TABLE IV

Adoptive Response of Spleen Cells from Mice Preimmunized with Z and Challenged with Z and AMEF

Group	Type of challenge	Titer							
		day 7		day 14		day 19		day 25	
		Act.	Prec.	Act.	Prec.	Act.	Prec.	Act.	Prec.
1	No challenge*	0.28	0.18	328	n.t.	48	40	101	n.t.
2	10 μg Z	1280	4.5	5040	n.t.	4570	330	2140	n.t.
3	10 μg AMEF	290	2.0	850	n.t.	704	330	720	n.t.

2×10^7 Spleen cells from donors preimmunized with Z were transferred to three groups of X-irradiated mice which were, respectively: not challenged, injected with Z, and injected with AMEF. The response in activating and precipitating antibodies during 25 days after transfer was followed. The units are the same as those used in Table I. Act., activating; Prec., precipitating; n.t., not tested.

* Residual primary response.

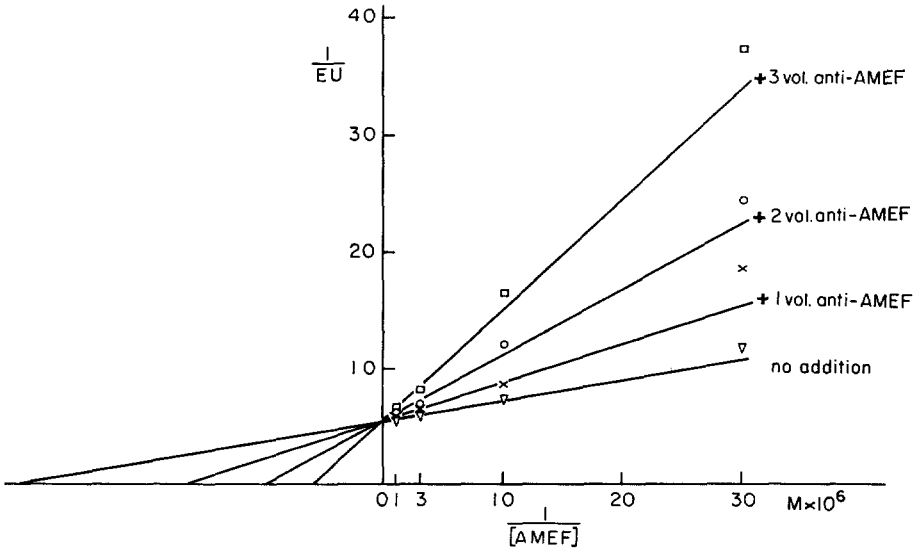

FIG. 2. Double reciprocal plot of enzyme activity as a function of AMEF concentration in the presence of a fixed concentration of anti-Z serum and various concentrations of anti-AMEF serum. *Triangles*: 1700 precipitating units (P.U.) of anti-Z (1 P.U. is defined as the amount of serum necessary to precipitate 1 EU of Z, taking 50% precipitation as the end point). *Crosses* (×): 1700 P.U. of anti-Z plus 250 P.U. of anti-AMEF. *Open circles*: 1700 P.U. of anti-Z plus 500 P.U. of anti-AMEF. *Squares*: 1700 P.U. of anti-Z plus 750 P.U. of anti-AMEF.

cipitating antibodies, although this was considerably smaller than that caused by Z.

Competition Between Anti-AMEF and Activating Antibodies.—Although anti-AMEF sera did not activate AMEF, they could still contain specific antibodies capable of competing with the activating antibodies present in anti-Z sera. This possibility was tested by mixing anti-AMEF and anti-Z sera in different proportions and then assaying each mixture for its ability to activate serial concentrations of AMEF ranging from 10^{-6} to 3×10^{-8} M. The results can be conveniently expressed in a double reciprocal plot of 1/EU against 1/[AMEF]. In Fig. 2 it is shown that under conditions of limiting AMEF concentration (points on the right side of the graph), mixing 1, 2, or 3 vol of anti-AMEF serum with a given amount of anti-Z serum resulted in progressively less enzymatic activity. In contrast, in excess of AMEF (points near the ordinate) no significant inhibition was seen. The family of rectilinear curves obtained extrapolated to the same value on the ordinate as expected if competitive inhibition was involved.

DISCUSSION

The results presented above show that AMEF and Z, two large polypeptides coded by a mutant and the normal allele of the β-D-galactoside gene of *Escherichia coli*, respectively, differ in their immunogenicity since only the latter can stimulate the production of antibodies capable of activating AMEF. On the other hand, the tests for cross-reactivity indicated no differences in the capacity to absorb antibodies directed against either of them. Assuming that a given site of the β-D-galactosidase molecule is endowed with both immunogenicity and antigenicity with regard to activating antibodies, it would follow that the determinant of Z responsible for eliciting the production of activating antibodies is also present in AMEF but in a somewhat altered conformation. This assumption is consistent with two lines of evidence, namely, (*a*) genetic data indicating that the difference in primary structure between AMEF and Z resides in one amino acid substitution (17), and (*b*) the binding of a single molecule of antibody or of Fab is enough to increase the activity of AMEF (18).[2] Under this hypothesis, one would expect that the altered determinant in AMEF would elicit antibodies which could bind AMEF but could not activate it. The fact that anti-AMEF serum competes with anti-Z serum for activation (Fig. 2) supports this idea.

The suppressive effect on production of activating antibodies caused by primary immunization with AMEF resembles that found in certain hapten-carrier experiments in which primary immunization with a given hapten-carrier molecule prevented an antibody response to a cross-reacting hapten when the latter was used as immunogen on the same carrier (24). This effect has been explained by the model of the "original antigenic sin," which postulates that a secondary

immunization with a cross-reacting determinant serves to stimulate a cell population which had been previously enlarged by injection of the original immunogen instead of reacting with naive specific antigen-sensitive cells. According to this theory, if one considers only the immunogenic determinant responsible for the synthesis of activating antibodies, our results could be explained by the primary injection of AMEF producing an increase in the population of cells which synthesizes nonactivating antibodies against the determinant. Upon subsequent exposure to the cross-reacting determinant of Z these cells would heighten their production of nonactivating antibodies. However, this would be an oversimplification since the results of our adoptive transfer experiments show the presence of antigen-sensitive cells specific for activating antibodies in animals which exhibited a suppressed response to Z. Thus, the suppression effect of AMEF sensitization could be attributed to both circulating antibodies and memory cells competing with naive antigen-sensitive cells for the newly injected Z. In addition, since the anti-AMEF antibodies can compete with activating antibodies for AMEF, the presence of small proportions of activating antibodies in the serum of animals exhibiting AMEF-mediated suppression would tend to be undetectable. From these considerations, one would predict that immunogenic blanketing between determinants which are closely related will be frequently found among sets of complex macromolecules differing in the make-up of one determinant while identical for many others. In order to test this prediction, it would be expedient to search for more pairs of macromolecules such as AMEF and Z in which a single determinant can be specifically and easily assayed.

According to an alternative model, a given class of antigen-sensitive cells would be potential responders to *both* Z and AMEF. The first of these two molecules to come in contact with the cell would determine the final three-dimensional configuration (in terms of ability to activate) of the antibody determinant produced by the cell. This idea agrees with the framework of theories on antibody formation which postulates that oligopotent cell precursors of antibody-forming cells have information for several amino acid sequences: for example, a discrete number of the corresponding DNA segments would be inserted in the gene coding for the variable region of the immunoglobulin chains. During the induction phase the antigen may play a role in this choice, thereby modulating the binding characteristics of the antibody that the cell clone will eventually produce (25).

If anti-AMEF antibodies bind AMEF at the same site as anti-Z–activating antibodies, a molecular mechanism is needed to explain why the anti-AMEF sera fail to elicit the β-galactosidase function of AMEF. A plausible explanation is illustrated in Fig. 3, using a key-keyhole symbolism. The square, round, or triangular figures represent the position of binding forces within the antigenic determinant; we shall call them here *a*, *b*, and *c* in their protruded form and

α, β, and γ in their invaginated one, respectively. Choosing this number of sub-sites is arbitrary. In fact, the combining region of antibodies directed

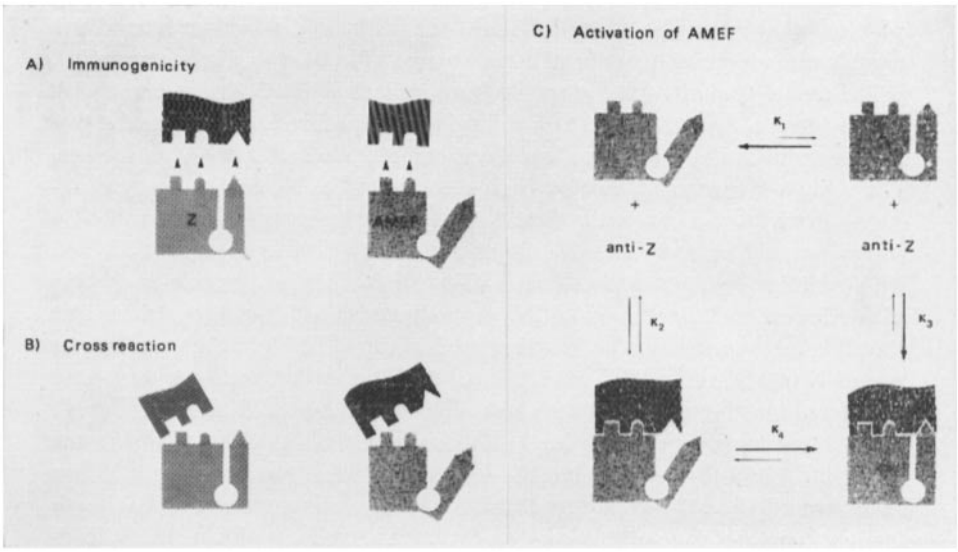

FIG. 3. A model consistent with our findings on immunogenicity and antigenicity of Z and AMEF and on AMEF activation. The units represent determinants in either Z, AMEF, or the respective antibodies using key-keyhole nomenclature to indicate chemical groups in each determinant. Units with invaginations represent antibody determinants, and those with protrusions, antigen determinants.

Fig. 3 A: Immunogenicity. The difference in conformation between Z and AMEF is symbolized by the displacement of one of the groups (the triangular-shaped group on the right side). As a consequence, Z and AMEF elicit different antibodies (dark cross-hatched area) directed against the site in question.

Fig. 3 B: Cross-reaction. AMEF, Z, anti-AMEF, and anti-Z antibodies fully cross-react in all combinations due to the presence of at least two groups in common. AMEF competes with Z for anti-Z antibodies.

Fig. 3 C: Activation of AMEF. Alternative model 1: AMEF exists in two forms, one enzymatically active, the other inactive, which are in equilibrium with a constant K_1 favoring the inactive form. The presence of activating antibodies displaces the equilibrium to the right by binding preferentially to the active form (with three dots) in a reaction with K_3 as constant. Alternative model 2: AMEF exists as a single molecular species. The activation by antibodies proceeds in two steps with K_2 and K_4 as constants, respectively. An allosteric reaction of AMEF with activating antibodies causes a conformational change in AMEF resulting in a three-dimensional structure similar to Z. The anti-AMEF antibody does not lead to this allosteric change.

against "sequential" determinants of polypeptide nature has been recently shown to accommodate up to four amino acid residues (28), while a "conformation-dependent" determinant may involve many more sterically related

residues (23). Both circulating antibodies and cellular receptors specific for Z would have determinants with α-β-γ components, while their counterparts specific for AMEF would only have α-β. The antigens would both have all the *a-b-c* components except that, in AMEF, one of them, *c*, would be altered but capable of undergoing a conformational change so as to resemble the normal *c*.

The model accounts for our results by postulating that a determinant with only *a-b* has a low but finite probability of stimulating a cell with an α-β-γ receptor while still capable of absorbing completely humoral α-β-γ antibodies. Thus, the *a-b* determinant could elicit α-β antibodies efficiently but only reduced amounts of α-β-γ antibodies. Cross-reactions between the *a-b* and *a-b-c* antigens would be complete with either α-β or α-β-γ antibodies (Fig. 3A and B). According to the model, the activation of AMEF is a protein-to-protein allosteric reaction directed by anti-Z antibodies with α-β-γ configuration. Two plausible mechanisms for this reaction are illustrated in Fig. 3C. According to one of them, in the AMEF, there is an equilibrium between enzymatically active and inactive molecules with an equilibrium constant K_1 favoring largely the inactive form. The activating antibodies would bind preferentially to the active form and therefore would displace the equilibrium towards the active form. According to the second mechanism, the activating antibodies react with a single molecular species of AMEF and cause the conformational change leading to an increase in enzymatic activity (bottom part of Fig. 3C). The γ component of the antibody would be required in the activation reaction in order to produce an induced fit. Considering the fact that AMEF is more negatively charged than Z, one could visualize γ as a charged group which would neutralize the negative component of the AMEF determinant. This idea is supported by the finding of two types of antibodies which differ both in their electrical charge and in the ability to activate different genetically altered β-D-galactosidases (26).

The postulate that the activation of AMEF occurs through an allosteric reaction is consistent with the observations that the *E. coli* mutants containing altered β-D-galactosidases activable by anti-Z serum map throughout the genetic region determining the structure of the enzyme (12, 17, 27), since it rules out the possibility that the catalytic site of the enzyme is directly involved.

SUMMARY

Two closely related protein antigens were used to study immunogenic competition. Namely, normal β-D-galactosidase of *Escherichia coli* (Z) and a genetically defective β-D-galactosidase (AMEF) which seems to differ from the normal in one amino acid substitution. A unique characteristic of this pair of antigens is that, although they are indistinguishable in precipitation and absorption tests with antibodies, the enzymatic activity of AMEF is specifically increased several-hundredfold in the presence of antibodies directed against Z. The fol-

lowing results show that Z and AMEF also differ in their immunogenic ability: (*a*) antibodies directed against Z activated AMEF; antibodies directed against AMEF did not activate, but competed specifically with activating antibodies. (*b*) Animals immunized with AMEF failed to produce activating antibodies when they were subsequently challenged with Z, although the presence of some cells primed to produce activating antibodies could be demonstrated by adoptive transfer. (*c*) Animals preimmunized with Z were stimulated in their production of activating antibodies by AMEF challenge, although not as efficiently as with Z.

A model explaining these observations by competition for the immunogenic site among antigen-sensitive cells carrying cross-reacting receptors is presented.

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