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Antibody-mediated enzyme formation: Its legacy at age fifty-four

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Abstract

Antibody-mediated enzyme formation is a phenomenon first described in 1968 and further studied by molecular Immunologists and Biochemists over the following five decades. The present review is made mainly by analyzing the 27 articles concerned with AMEF that appeared over the course of 47 years, commenting 16 original figures selected to be re-printed in AMEF's Legacy. We, the reviewers, started by revisiting our own "insider's" experience of discovery, and followed by considering all results, our own and of members of other AMEF Labs. We had planned to conclude the review by correlating the various AMEF mutants to a detailed knowledge of the consensus betaGal structure. However, we became aware of several "robust" papers, published between 1989 and 2014, by authors outside of AMEF Labs. We familiarly called this surge: "The Second Wave" and adorned it with a doodle in Hokusai style. We were thrilled and happy to take them on board and properly examined their data. A team of this second wave had imagined unique uses for AMEF, and new doors to modern biotechnology. Another one had used AMEF as Tool and Marker to attain high levels of crystallography, solving puzzles of conformation, and ultimate structure. Together, they doubled our motivation to review AMEF. Serendipity gives us back the pleasure of finding, a treat at any age.

KEYWORDS
activation, antibody, beta galactosidase, conformation, flexibility, mutation, paratope

1 | INTRODUCTION

In 1966 two researchers, an Immunologist and a Biochemist, met at Karolinska Institute in Stockholm and collaborated on the application of fluorochromasia on HLA (Human Leucocyte Antigen) Typing. The two also discussed the possibility of restoring the catalytic activity of a mutant enzyme molecule by exposing it to an anti-wild type enzyme antibody. The scheme looked like a shot in the dark, nevertheless in June 1967 the two friends convened again at Brown University, Providence (RI, USA) and set up an experiment where

antibodies elicited against the β-galactosidase (GZ) enzyme from wild type *Escherichia coli* were added to defective (almost inactive) betaGal molecules from *E coli* strains carrying missense point mutations in the lacZ region. The result was a clear success: the defective betaGal from one of the mutant strains reached a 500-fold enhancement of activity. This phenomenon was called AMEF, acronym of antibody-mediated enzyme formation.

Fifty-three years later, a combination of two improbabilities, the free time suddenly allotted to the hostages of the coronavirus pandemic coupled with the persistent curiosity in aging finders and witnesses of the discovery of AMEF, prompted the same Immunologist, Franco Celada, together with a somewhat younger Biochemist,

Roberto Strom (who had participated in most of the refinement studies during the decade following the first result), to undertake a re-visitation of AMEF history and of its significance. The aim of this review is to commemorate the birth of a new use of specific antibodies as TOOLS to re-activate the betaGal enzyme made defective by a missense point mutation and to illustrate the new developments triggered by AMEF as well as the brilliant and unsuspected inventions and unveilings, which come as late cherries to the AMEF saga.

As insider reviewers, we had to pledge total objectivity, and our advantage was the enjoyment of a constant flow of personal memories about experiments, results, facts, and other events, which, if allowed to enter the text, would decrease the distance between Life and Science, and would lighten the scientific reading.

2 | INTERDISCIPLINARITY

At a Nobel Symposium on SYMMETRY (in Stockholm in the 60's), Celada heard two *Nobel laureates* exchanging these spirited remarks:

"I - said Monod - speak to the Immunologists!"

"I - said Crick - do not speak to the Immunologists, but the Immunologists speak to me!"

Celada laughed with the audience at this exchange, but he also took it as a precious advice to be an interdisciplinarian, working with Biochemists, Geneticists, Enzymologists, Semiologists, Astrophysicists, and cooperating with mathematicians to build a computational modeling of the immune system.

3 | THE ORIGIN

1966, Stockholm. Franco Celada, a recent Docent at Karolinska Institutet, is looking for the highest sensitivity method that would facilitate his work[1] on adoptive memory cell cultures. He is hoping that, with a method sensitive to single molecules, he may succeed to disprove the idea that long-term Memory should require the presence of antigen in the memory cell. Cinader,[2,3] had recently examined several enzymes, whose activity was modified by specific antibodies; only few of them, as described, for example, by Pollock,[4] exhibited a small increase of activity, certainly not sufficient for a precision measuring. To clear his mind, Celada has taken an appointment with Boris Rotman, a Chilean Biochemist who is spending a sabbatical at Karolinska before moving, as a newly appointed professor, to Brown University (R.I., U.S.A.). Celada and Rotman are already collaborating: a manuscript[5] proposing the use of fluorochromasia in HLA typing has just been submitted.

Here is an extract of their lunch conversation:

FC: Congratulations for your seminar last week: those droplets containing one molecule of enzyme were fantastic! Now, regarding my adoptive memory cultures, my question is, how can one measure a single antibody molecule, or a single Ab +Ag event?

BR: Only if the antibody has enzymatic activity! Have you heard about such antibodies?

FC: No, I have not. Let me now consider enzyme molecules that are inactivated by specific antibodies, by picking one of Cinader's enzymes and using it as antigen. The antibodies will be measured by the decrease of enzyme activity ... but most of the time the signal will be calculated against a high background. I would greatly prefer a quite opposite design, where the free antigen, untouched, would have no activity – and where the antibody would light up the enzyme activity, by its specific binding! A firefly in a dark forest, or a star in a moonless night!

Tell me, Boris, is this feasible, or incurably crazy?

BR Perhaps one could modify the Ab to introduce β-galactosidase activity. But I do not know how that can be done. It is not feasible at present. Perhaps one could find a mutant of β-galactosidase without enzymatic activity that will become active in the presence of antibody.

FC: How difficult is it to find such a mutant?

BR: It can be extremely difficult, but Van Niels said: "If a reaction can exist in nature, one should find a bacterium that carries it." There are several bacterial strains with mutated inactive β-galactosidase, and I can obtain some samples from Lederberg's lab in Stanford, CA.

FC: "Aren't we forgetting something? What are the probabilities of finding an *activable* mutant?"

Boris answered:

BR: "Nearly zero, but not zero. Nothing is impossible in biology. Anyway, I like nearly impossible projects."

FC: Let's try it! If we succeed, we shall spot fireflies in a moonless night.

Boris remained skeptical about the feasibility of the experiment, because there was no precedent for any similar effect of antibodies (at the time he was not aware of Pollock's experiments) - but Celada's enthusiasm convinced him to give it a try.

4 | THE ABDUCTION

A review should make order and help connect with the object presented. The AMEF legacy begins with the formulation of a hypothesis (called *abduction* here, in Peirce's mode) sometime in the autumn of 1966 in Stockholm and continues as a two-month-collaboration leading to a discovery in Providence, conveniently located in the Narragansett Bay, in full sailing season. The nine-month interval phase was not used for any preparation except the immediate adoption of Rotman's suggestion to use point-mutant, inactive betaGal as target. This completed their abduction: it was never actually written, but I can transcribe it from memories and talks of the two.

Imagine two doctors standing in front of the patient, a "mutant," with a tentative diagnosis and only one therapy, never tried before. Here is the abduction:

"*A single mutation hits a spot and alters an unknown number of equilibria of the betaGal conformation, causing the enzyme to become active.*"

The two doctors say:

> We bet our money that this specific antibody is a cure for the mutant. If it manages to bind to the mutant, the antibody will do its best to improve its grasp by convincing the epitope to come back to its original wild-type (wt) shape: the steps in this direction are energetically virtuous, and it is feasible for the entire conformation to snap back to wt, and thus, to function.

5 | THE 9-MONTHS HIATUS

A 9-months interval was used by Rotman to set up his new Lab at Brown University, and by Celada to publish his paper[1] on Memory Cell Adoptive Transfers and, immediately afterward, to take his wife and their three children to the Arlanda Airport, to wave them off to a vacation in the Alps, ship his Volvo to the harbor of New York, from where he would pick it up and drive to Rhode Island on June 2. The shipping of a two-year-old car on the Volvo-cargo vessel is part of a Swedish scheme to avoid taxes, cost 100 dollars and it worked great.

6 | DISCLAIMER

We will not discuss why the elder of two colleagues arranged a meeting with Geneticist Seymour Lederberg of Brown University the day before the experiment, to evaluate the sanity of our project. For the record the answer was: "If you two guys can think of it, the Phenomenon exists. To find it, depends on Probability." (cf. Saint Anselm's proof of the existence of God: "If the greatest being exists in the mind, it must also exist in reality").

7 | THE 1967 EXPERIMENT

The newly built laboratory in Brown University, Rhode Island, was large and had working rooms at $-20°C$, $+5°C$, and $+37°C$. Franco Celada joined Boris Rotman and his assistants, Rosario Guzman (from Chile) and John Ellis. We were going to test 47 different *E coli* strains carrying point mutations in the lac Z gene (and produced, therefore, mutated betaGal proteins with a very low level of enzymatic activity), strains that Boris had just received by mail from Esther Lederberg in California. We planned to grow the cultures overnight, centrifuge, separate the sediments, pass them in the French press (characteristically noisy but efficient) to break all bacterial cells, distribute them into large tubes in 10x4 racks, then add rabbit serum that contained anti-betaGal antibodies (or control serum, or no serum), incubate for 1 hour, add *o*-nitrophenyl galactoside (ONPG), and measure, in a colorimeter, the orange color produced by the betaGal catalysis.

The work was relatively simple, but it had to be re-planned because of the exceedingly high number of tests to be performed on the 47 different bacterial strains. Fortunately, none of the members of the team was in a hurry to leave. We decided to prolong our stay without changing the rhythm: a sustainable number of three tests per day, three testing days *per* week; the testing went on for 6 weeks, allowing some re-testing and more necessary controls. By the end of July, we were done with the experiments, the figures needed for an article, and many, enthusiastic discussions. During all week-ends of July and August, there were seven-class regattas in Narraganset Bay: Boris was tactician on a noble Herreeschof-S, and Franco was crewing on an Ensign.

8 | THE DISCOVERY: FIRST EXPERIMENTAL RESULTS AND THE FULFILLMENT OF THE ABDUCTION

Thirty-seven bacterial strains (out of 47) showed no enhancement of the basal enzymatic activities of their betaGal defective enzymes. In six of them, the enhancement factor was between 2% and 14%, two had enhancement factors 9 and 43, one was over 500. None of us, who were in the lab on that June 13, will ever forget the moment when strain W1601 produced an explosion of color and was named "AMEF" (it will be become, later on, "AMEF#6101").

This first experiment in a new field was yielding thrilling results, but needed refinements. Anti-wt.GZ antibodies had caused a strong activation of betaGal activity in the AMEF extract, and the team knew, before the third week expired, that their ABDUCTION was confirmed. The relatively low (but measurable) basal betaGal activity contained in freshly prepared AMEF extracts appeared to be associated, when examined in a sucrose gradient, to a macromolecule with a sedimentation pattern very similar to that of the tetrameric wtGZ enzyme. A reasonable point to be investigated that was raised in our early discussions, would have been to check whether anti-AMEF antibodies exerted any effect on the betaGal activity of the W1601 strain - but 3 months were needed to raise these antibodies. In addition, other points, in our opinion, needed further clarification: (a) the size of the minimal protein unit (monomer, dimer, or tetramer?) susceptible of being activated upon interaction with anti-wtGZ, (b) the effective minimal paratope-to-protein ratio that could, in our AMEF preparation, lead to full activation of its latent enzymatic capability. The results of our first experiment seemed to us, anyhow, to be worth, *per se*, of more detailed examination and evaluation.

Figure 1 is the first image of the results, obtained in June 1967 at Brown University upon measurement of the levels of betaGal enzymatic activity in pairs of AMEF-containing parallel tubes after addition (at time 0, but alternatively also after 30 or 60 minutes) of anti-wtGZ antibody or of control serum). All tubes were measured for enzyme activity at 5 or 10 minutes intervals.

Celada remembers how, just by watching what happened in the assay tubes after addition of the ONPG betaGal substrate, he had two iconic impressions, one being the sheer magnitude of the antibody-dependent activation of ONPG hydrolysis, the other being, in controls, the surprisingly high background activity at time 0 that decayed to traces in about 1 hour, This experiment led in fact to two distinct findings:

1. Anti-wtGZ antibodies activated the mutant betaGal, a reaction that reached its apex in 30 minutes. The extent of the activation process was surprisingly high – about 10-fold in the first set of tubes
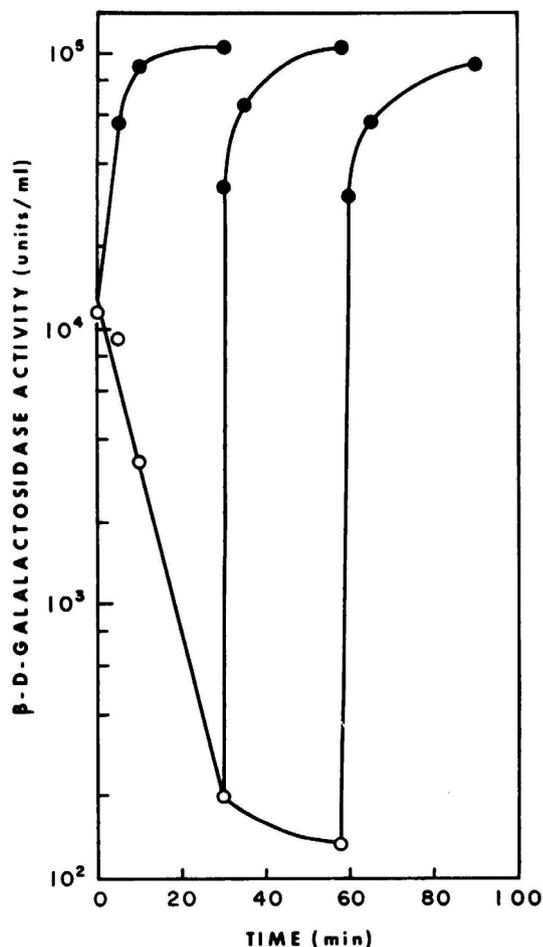
FIGURE 1 Development of betaGal enzymatic activity in the extract of AMEF#6101 after addition of anti-wtGZ antibodies (full circles) or of normal rabbit serum (fig. 3 by Rotman and Celada[6])

and becoming 550-fold when AMEF had been pre-incubated at 37°C for 30 minutes.

2. The "second result" was seen in the tubes that were considered controls. These tubes, taken from the refrigerator and transferred to 37°C, showed a relatively high level of basal catalytic activity that, however, rapidly decreased upon more prolonged exposure to 37°C and dropped to about 1% of the initial value after 60 minutes. This peculiar temperature dependence of the basal catalytic activity suggested (without, however, proving it) that the AMEF protein could undergo some conformational change that severely decreased its basal enzymatic activity without, however, modifying the ability of anti-wtGZ to stimulate a full activation of that defective *lac*Z gene product.

9 | WAITING FOR PUBLICATION

9.1 | The "kidnap" to West-Berlin

In late August 1967, Franco Celada had flown from Boston to London in order to catch the connection to Stockholm. In the Heathrow waiting

lounge, he met another Immunologist, Fritz Melchers. The two had met once in California, where Fritz was staging at the Salk Institute, and Fritz gave the impression of high competence. In the enthusiasm of his recent discovery, Celada told him the entire story of AMEF. This, however, increased, instead of appeasing, Fritz's curiosity. He said: "Can't you fly with me to West-Berlin today? This afternoon you will give a talk to my group, mostly students: they will be thrilled to hear it from the horse's mouth, and by tomorrow midday, you will land in Arlanda !" Franco was trying to raise some objections, but Fritz was unstoppable, he immediately got the tickets changed through his office. It was nevertheless past 7 PM when they arrived at West-Berlin University. Celada was wondering about his oncoming seminar. Fritz introduced him to the audience, who timely informed him that all of them had skipped their suppers. All the students were taking notes, but many of them (obviously those educated in East Germany), were not fluent in English. Celada felt almost obliged to give the seminar in his broken German, although it was for him a real effort.

9.2 | The Roma connection

A few months later Franco Celada contacted his close friend Valerio Monesi, a brilliant Cell Biologist that he had met in 1959-61 when both of them had spent two very fruitful years in the Biology Division of the Oak Ridge National Laboratory. Monesi had returned a few years before to Italy at the Casaccia Laboratory (near Rome) of the Italian National Center for Nuclear Energy, but in 1968 he was on the verge of being nominated as full professor of Histology and Human Embryology at *La Sapienza* University of Rome. Upon learning about Celada's results on AMEF, he presumed that they could be of interest to Professor Alessandro Rossi Fanelli, who was well known, with the members of his team - among whom, in particular, Eraldo Antonini - for their scientific contributions to the structure-function problems of hemoglobin and of enzymatically active proteins. Celada was, therefore, invited, in the first months of year 1969, to give a seminar at the Institute of Biochemistry of *La Sapienza* University of Rome.

Before the seminar, he got a tour of the laboratories of that Institute, particularly of the students' benches, where he met a young frizzy-redhead post-doc, Roberto Strom, who showed a very high interest in the discussion about AMEF.

The seminar was a success. As well as the members of the Institute of Biochemistry, the audience included one of its a frequent guests, the well-known expert of Protein Physical Chemistry Jeffries Wyman Jr., who, 3 years earlier, had elaborated, with Monod and Changeux, the allosteric theory of ligand binding to multimeric proteins. Here is his dialogue with Celada:

JW: Nice finding, congrats, what is next in your Karolinska lab?

FC: My anti-AMEF rabbit sera are almost ready to be tested: will they activate AMEF, or not? This is the question! Would you care to guess?

JW: No need to guess. Anti-AMEF will not activate, by pure reason.

As soon as Celada returned to Stockholm, he verified this prediction, finding that it was fully confirmed.

As a consequence of this seminar, Roberto Strom, the young post-doc of the Rome Institute of Biochemistry, joined Franco Celada in June 1968 at the Karolinska Institute in Stockholm: a long friendship began.

10 | THE AMEF LABS

Experimental work on AMEF continued for almost 15 years in three different laboratories. While Boris Rotman and his staff (Rosario Guzman and John Ellis) invited Alberto Macario and his wife Everly Conway de Macario to come to Brown University in Providence, Rhode Island, Franco Celada remained at Karolinska Institute in Stockholm for two more years, working with Roberto Strom and Kerstin Bodlund. He then decided to spend 1 year, starting from autumn 1970, at the Pasteur Institute in Paris, collaborating with Agnès Ullmann and Jacques Monod on the effects of anti-wtGZ antibodies on the ω-complementation of β-galactosidase. In 1971, he returned to Italy as "chief Immunologist" at the Italian Research Council in Rome; he worked at the Cell Biology Laboratory with Roberto Strom, Roberto Tosi, Roberto Accolla and Birgitta Åsjö for 5 years, then took a sabbatical year at University of California in Los Angeles (UCLA) working with Irving Zabin and with Eli Sercarz. In 1977, he returned to Italy, and settled in Genova University as full Professor of Immunology. In this lab, his collaborators were Jasna Radojkovic, Fabrizio Manca, Annalisa Kunkl, Antonio Lanzavecchia, Giuseppina LaPira, Caterina Cambiaggi, and Renata Cinà. During his wandering time, his lab changed addresses but kept the focus on AMEF alive.

The third independent group, located in Berlin University, was formed by Walter Messer (a bacterial Geneticist) and Fritz Melchers (an immunologist, who became, a few years later, Director of the Basel Institute). They had been directly informed about AMEF activation, through Celada's seminar in West Berlin, before the publication of the first paper, and joined enthusiastically and effectively the AMEF history.

The articles published on AMEF and mentioned in this review span from 1968 to 2014 for a total of 27; they are listed as "Amef-related" references, 24 of these articles (i.e. until year 1992) were directly aimed to the study of AMEF, while the last ones – namely references 36 (dated 1998), 37 (dated 2002) and 38 (dated 2014) reached the same goal by utilizing AMEF as a tool or as a marker promoting original ideas and advances in modern Biotechnology or in the highest levels of Crystallography.

11 | FURTHER INVESTIGATIONS, IN THE AMEF LABS, ON THE ACTIVATION PROCESS OF DEFECTIVE BETAGAL GENE PRODUCTS BY anti-wtGZ POLYCLONAL ANTIBODIES

In a meeting on The Lactose Operon, held in the late Spring of 1969 at the Cold Spring Harbor Laboratory (with written reports published in 1970 as CSH Monograph vol.1), identical activating effects on AMEF#6101 could be exerted, in parallel experiments, by preparations of divalent anti-wtGZ and by their Fab monovalent fragments obtained by digestion with papain. Upon centrifugation that caused selective sedimentation of the immune complexes cross-linked by the divalent antibodies, all the enzymatic activity of the samples treated with monovalent Fab fragments remained in the supernatant (Figure 2).

It was thus possible to discard the hypothesis that, in AMEF, the defective betaGal protein be activated through an antibody-induced cross-linking mechanism acting on its subunits. When increasing concentrations of AMEF#6101 are exposed to a fixed amount of anti-wtGZ antibodies (or of their Fab fragments), the final levels of activity follow a hyperbolic shape toward a horizontal asymptote (that can be converted to a straight line in a double reciprocal plot). The activation process seems to occur as a consequence of a basically simple 1:1 epitope-paratope interaction, but its kinetics is consistent with the presence of a monomolecular rate-limiting step occurring within the AMEF molecules.

In fact, shortly after the end of the Lactose Operon Meeting, Celada and Rotman could even show, by a similar procedure that their anti-AMEF antibodies were unable to activate AMEF#6101, and also inhibited, through a competition involving the same AMEF epitope, the activating effect of anti-GZ antibodies (Figure 3).
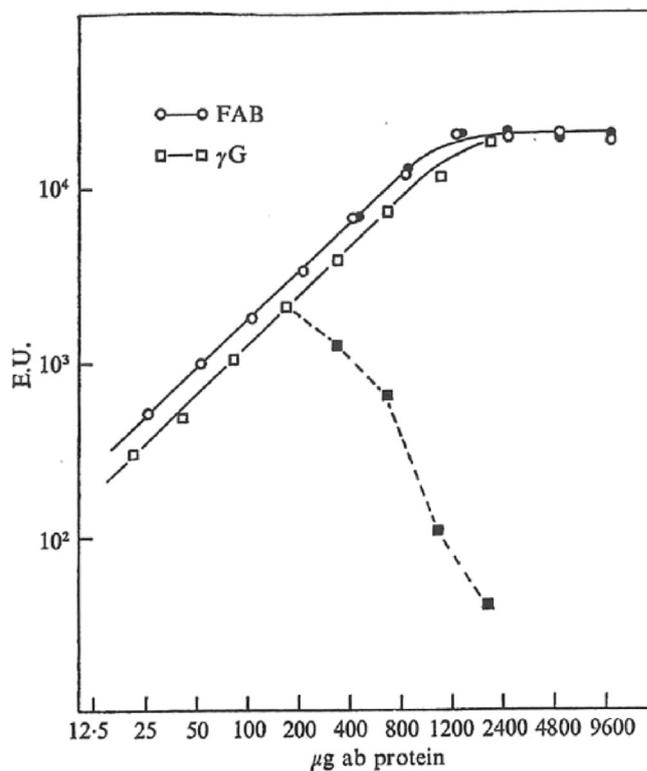
FIGURE 2 Dependence of enzymatic activity of AMEF#6101 upon addition of anti-GZ antibodies concentrations of divalent anti-GZ antibodies (full circles) or of their monovalent Fab fragments (empty circles) (fig. 2 by Celada et al[7])

Roth and Rotman[9] showed, some years later that these anti-AMEF antibodies were also non-competitive inhibitors of the catalytic activity of the wild-type β-galactosidase enzyme.

In the same Lactose Operon Meeting, the Berlin group (the immunologist Fritz Melchers and the bacterial geneticist Walter Messer) showed[10]
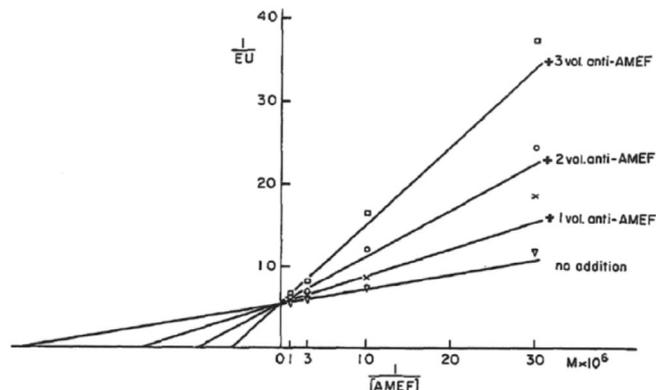
FIGURE 3 Anti-AMEF sera competition against activation of AMEF#6101 by anti-wtGZ antibodies. The enzyme activity is plotted, in a double reciprocal plot, against the AMEF concentration in the presence of various concentrations of an anti-AMEF antibody preparation (fig. 2 by Celada et al[8])

that the AMEF concept could be extended to several other defective *lacZ* gene products - produced by as many as 11 other missense point mutant *E coli* strains, whose genetic map is shown in Figure 4.

These other antibody-activable defective enzymes (that we will also call AMEFs) exhibited[11] non-uniform susceptibility to activation by anti-wtGZ antibodies: in AMEF#40 and AMEF#918 - products of strains carrying their point mutations at the distal end of the *lacZ* gene (the so-called "MM.group 2") - the level of the "activation factor" was around 50×, while it was definitely higher (over 250×) for all the other AMEFs (except for AMEF#950). It was later shown by the same research group[12] that all their 11 AMEFs had, in their native state, a tetrameric structure similar to that of wtGZ, that is, formed by association of 4 subunits whose molecular mass of 130 ± 15 kDa.

These AMEF protomers could even combine with wt-GZ, forming hybrids (Figure 5) whose enzymatic activity was, however, found[13,14] to be proportional to the number of wt-GZ protomers.

Association of betaGal monomers (wt-GZ as well as AMEFs) in tetrameric structures appears, therefore, to be a necessary but not sufficient condition for the acquisition of a high level of enzymatic activity by each protomer. In the very first 1967 experiment,[6] in fact, the freshly prepared (but enzymatically defective) AMEF#6101 had already been shown to be in a tetrameric form that lost, however, most of its basal activity upon incubation at 37°C. A later sucrose
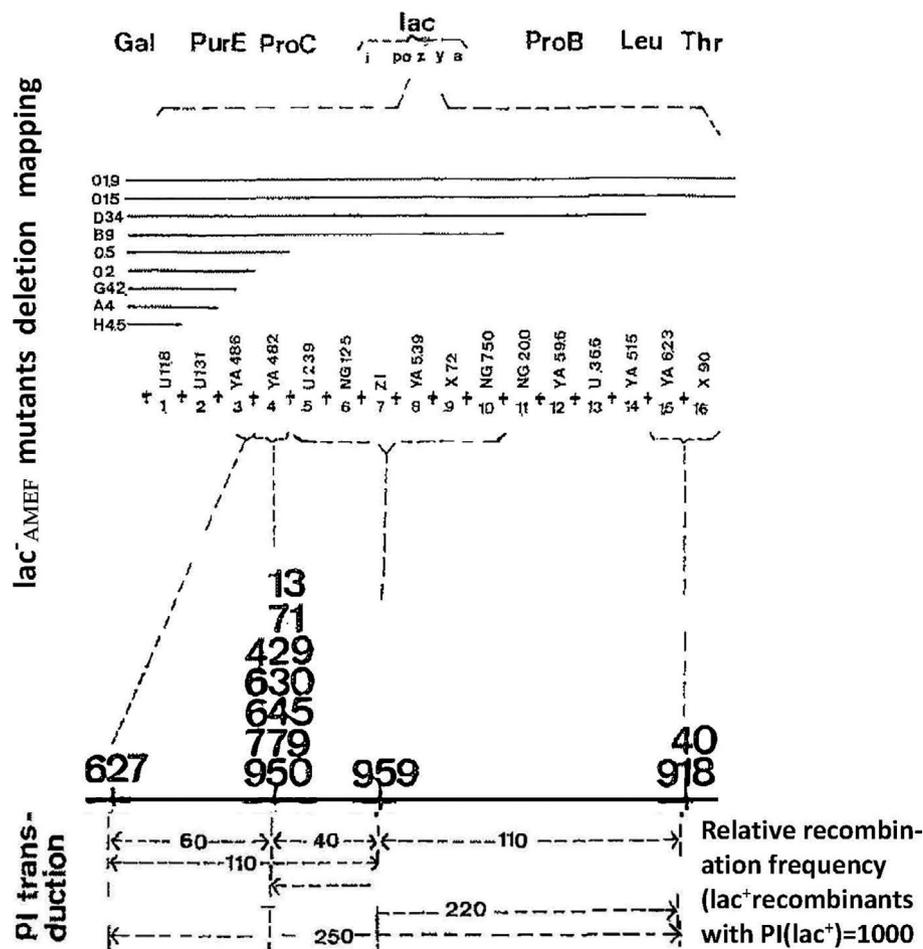
FIGURE 4 Genetic map of the Berlin defective point mutants (fig. 1 by Messer and Melchers[10]). The position of the lacz gene in the E.coli genome is shown on the top line, followed in the top line, followed by a scheme of its deletion mapping. The central part of the figure indicates the positions of of the various Berlin defective point mutants estimated from their relative recombination frequencies in PI(lac+) assays
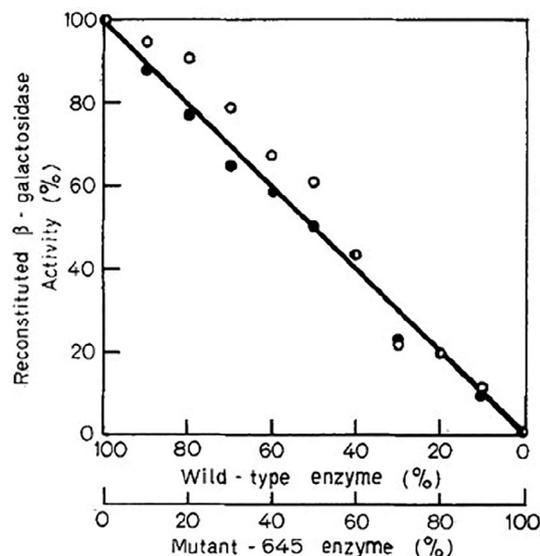
FIGURE 5 Enzymatic activity of wtGZ-AMEF#645 hybrids having different GZ/AMEF ratios. Empty circles and full circles indicate the situations immediately after hybrid formation and 7 days, after, respectively (fig. 1 by Melchers and Messer[14])

FIGURE 6 Sucrose gradient centrifugation of a ^{14}C-labeled AMEF#6101 preparation in the absence (empty circles) or presence (full circles) of antibody Fab fragments. The Fab fragments had been prepared from anti-wtGZ antibodies (panel A) or from normal immunoglobulins (panel B) (fig. 4 by Conway de Macario et al[15])

TABLE 1 Variation in activating antibody affinity during the primary immune response in vivo

Time after primary immunization	Activating titer (Enzyme units/μL)	$K_o \times 10^5$ M^{-1}	Heterogeneity index of the Sips distribution
11 days	3.01	4.83	0.9
68 days	6.50	9.30	0.8
8 months	0.75	32.60	1

Source: Modified from table 3 by Celada et al.[17]

gradient centrifugation analysis by Rotman's group[15] at Brown University in Rhode Island showed in fact (Figure 6) that these almost inactive AMEF#6101 macromolecules were essentially in a dimeric form that could be reverted to a tetrameric one upon interaction with anti-wtGZ Fab fragments.

Most of this inactivation process could, however, be prevented, or reversed,[16] by adding to this AMEF a betaGal substrate analog. As already shown in Figure 1, anti-wtGZ antibodies produced almost identical activation levels when they interacted with either form (tetrameric or dimeric) of AMEF#6101.[6] In both cases, this interaction definitely stabilized a tetrameric structure of the enzymologically activated AMEF.[15]

The anti-wtGZ antibodies obtained at various times after immunization of a donor rabbit differ in terms of their ability to activate a same AMEF. By assuming a Sips distribution for the samples containing non-homogeneous populations of anti-wtGZ antibodies, the mean values of their association constants for AMEF#6101 vary (Table 1) from 4.83×10^5 M^{-1} for the very early antibodies to over 32×10^5 M^{-1} for those taken 8 months after immunization.[17]

Although the unimodal Sips heterogeneity index has been shown by Bruni et al[18] to be inadequate for a full characterization of the affinity distribution of antibody populations raised during an immune response, these results indicate anyhow that most of the early antibodies have, toward AMEF#6101, an affinity constant much lower than those that are synthesized at later times.

In our early experiments,[7] the kinetics of the activation process of AMEF#6101 elicited by addition of anti-wtGZ antibodies was, as previously mentioned, consistent with the presence of a monomolecular rate-limiting step. This was found to be true also for AMEF#645,[19] with first-order rate constants having always the same value – around 1.0 hour^{-1} at 30°C – even upon wide variations of AMEF concentrations or of anti-wtGZ antiserum dilutions. In a later detailed analysis[20] of AMEF#6101 activation, we could instead detect a relatively wide

range of these first-order rate values that anyhow asymptotically converged, when activation was highest, toward a value somewhat higher than 2.0 hours^{-1} at 25°C.

The monomolecular rate-limiting step of the antibody-induced activation process may, however, be generated by AMEF interaction with anti-GZ paratope or pre-exist in AMEF itself. Experiments with Sepharose-bound activating antibodies have indeed shown[20,21] that both pathways do exist simultaneously, at different extent depending on the experimental conditions (Figure 7).

12 | EFFECTS OF anti-wtGZ ANTIBODIES ON BETAGAL COMPLEMENTATION

In 1963, during a Cold Spring Harbor Symposium on "Synthesis and Structure of Macromolecules," Perrin[22] had shown that, in *E coli* mutants characterized by partial deletions of their β-galactosidase structural gene, the betaGal enzymatic activity could be restored in the "acceptor" (inactive) protein by "complementation" with some
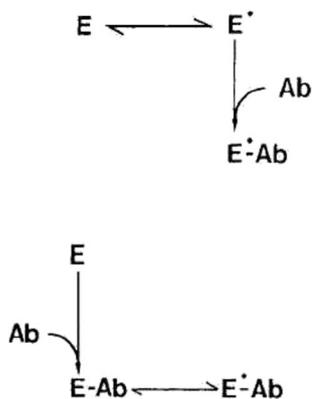
FIGURE 7 The two different pathways of antibody-mediated enzyme activation (fig. 3 by Celada and Strom[21])

"donor" peptide sequences encoded by the deleted segments of the *lac*Z gene. Two distinct α- and Ω-complementation systems were indeed described as occurring, respectively, at the N-terminal[23] or at the C-terminal[24] regions of the betaGal protein.

Accolla and Celada[25] found that, upon addition of moderate concentrations of anti-wtGZ antibodies to the "α-acceptor" *del*M15 protein (produced by an *E coli* strain with a large deletion of the operator-proximal portion of its lacZ gene), this protein acquired a rather relevant β-galactosidase activity (Figure 8A). As shown in Figure 8B and in Table 2, this effect was, however, at his maximum, only 20% of the enzymatic activation produced[23] by the α-complementing "donor" peptide - while addition of higher amounts of anti-wtGZ antibodies caused a loss of enzymatic activity even in the presence of the α-complementing peptide.

At low saturation values of *del*M15 by the α-complementing peptide, addition of anti-wtGZ antibodies causes an increase of betaGal enzymatic activity, which is not only larger than the sum of the effects caused by each of these "activators," but is also characterized by an increase in the rate of activation (Figure 9). Anti-wtGZ antibodies are, therefore, capable not only of "facilitating" the activation of *del*M15 by low amounts of the α-complementing peptide, but also of "accelerating" this process.

In the Ω-complementation system described in detail by Ullmann et al,[24] preliminary experiments had shown[26] that anti-wtGZ antibodies failed to exert any effect on the β-galactosidase activity induced by limiting amounts of an Ω-donor added to an excess of Ω-acceptor. Unexpectedly, however, in the presence of an excess of Ω-donor,[27] the same antibodies markedly increased (possibly through an "Ω-donor recruitment effect") the overall yield of Ω-complementation (Figure 10) - whose time course remained, however, unchanged.

13 | ENZYMATIC ACTIVATION OF DEFECTIVE BETAGAL PROTEINS BY MONOCLONAL anti-wtGZ ANTIBODIES

In October 1981, a European Molecular Biology Organization (EMBO)-sponsored meeting on "Protein Conformation as an Immunological Signal," was convened by Franco Celada in collaboration with
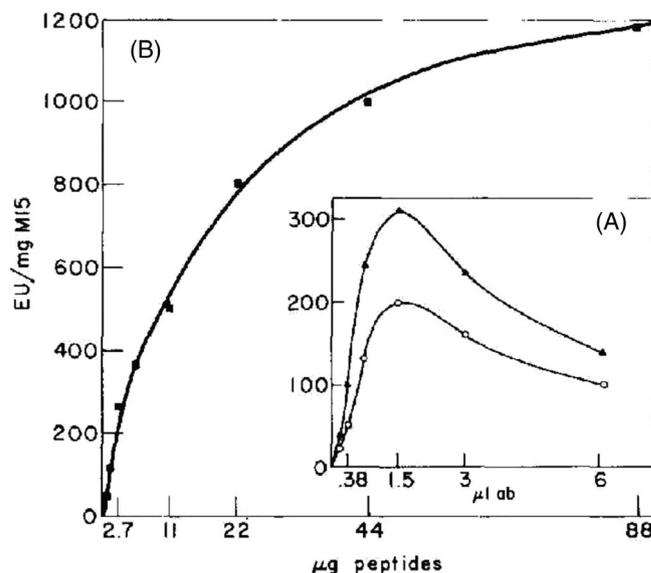
FIGURE 8 Panel A reported the enzymatic activation of delM15 by increasing concentrations of divalent anti-wt GZ antibody (empty circles) or of the corresponding Fab monovalent fragments (full circles). Panel B shows the activation curve obtained upon addition, to delM15, of the α-complementing peptide at increasing concentrations (fig. 1 by Accolla and Celada[25])

TABLE 2 Effect of different combinations of anti-wtGZ antibodies and of α-complementing peptide on the activity of a fixed quantity of the delM15 (α-acceptor) protein

Relative antibody concentration					
Relative peptide concentration		0	1	16	128
	0	<1	5	120	25
	1	18	28	140	23
	8	220	250	340	170
	128	2000	2000	1600	700

Source: From table 1 by Accolla and Celada.[25]

Verne Schumaker and Eli Sercarz. Within a topic focused on the changes in antigen conformation induced by specific antibodies, Roberto Accolla reported the results[28,29] that, in collaboration with R. Cinà, E. Montesoro, and F. Celada, had been obtained by using anti-wtGZ monoclonal antibodies present in the culture fluids of three different hybridoma clones (generated by somatic cell fusion a myeloma cell line with spleen cells from mice immunized with wtGZ). These three different monoclonals that had been generated by somatic cell fusion on a myeloma cell line with spleen cells from mice immunized with wtGZ, had been selected for their ability to "activate" AMEF#6101, but the level of activation was quite different under comparable experimental conditions: less than 2-fold for ZL.1-1b, almost 4-fold for ZL.2-1b, and over 15-fold for ZL.2-2.

When the three monoclonals (previously labeled by adding ³H-leucine to their hybridoma cultures) were tested for their ability to compete with each other for wtGZ-coated wells of a polyvinyl plate, the antibodies from clone ZL.2-2 were found to exert a very strong competition
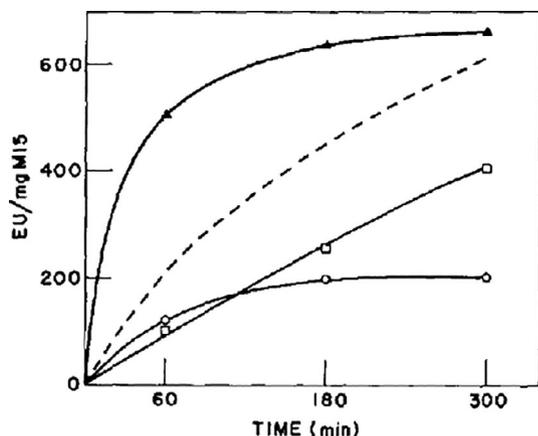
FIGURE 9 Acceleration by anti-wtGZ of the kinetics of α-complementation. The enzymatic activity acquired by a fixed quantity of delM15 was measured at different times after: (a) addition of an amount of anti-wtGZ antibody (empty circles) capable of producing optimal activation; (b) addition of a limited (non-saturating) amount of α-complementing peptide (open squares); (c) simultaneous addition (full triangles) of anti-wtGZ antibody and of α-complementing peptide in the same amounts as in (a) and (b). The broken curve is the sum of the effects produced separately by (a) and (b) (fig. 2 by Accolla and Celada[25])
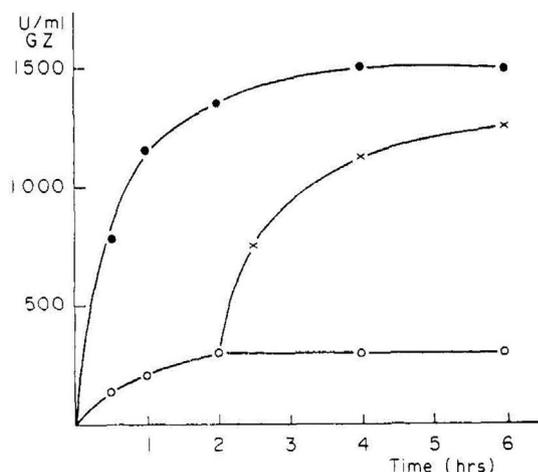
FIGURE 10 Ω-donor recruitment effect by anti-wtGZ antibodies on Ω-complementation of betaGal truncated mutants. The Ω-complementation reaction was performed by adding the "Ω-donor" protein from E coli strain W4680 to the "Ω-acceptor" protein from E coli strain S9080. In panel A, β-galactosidase activity was measured, in the presence (full circles) or absence (empty circles) of anti-wtGZ antibodies, starting immediately after addition of the Ω-acceptor and of a 6-fold excess of Ω-donor. Alternatively, the antibody was added, 2 hours after the onset of Ω-complementation reaction (crosses) (fig. 3 by Celada et al[27])

toward the antibodies synthesized by the other two clones. The reciprocal competition between antibodies of clone ZL.1-1b and those from clone ZL.2-1b was instead almost negligible (Table 3).

The paratopes of antibodies ZL.1-1b and ZL.2-1b seem, therefore, able to interact with AMEF#6101 at distinct, though presumably adjacent, epitope-like regions of this defective betaGal protein. AMEF#6101 activation by ZL.2.2. appears instead to involve both these regions, with an overall binding affinity value of approximately 5×10^6 M^{-1}. It was, however, found that this same ZL.2.2. monoclonal has an almost 200-fold higher binding affinity ($K_a = 9.6 \times 10^8$ M^{-1}) for the active wtGZ enzyme.

Since every AMEF is a defective protein generated by one missense point mutation of its coding sequence, it can hardly be the difference between these affinity values, which can hardly be ascribed to the loss, in the AMEF defective protein generated by a single missense point mutation of its coding sequence, of several epitopes present in the wild type enzyme. It could perhaps be due to the presence, in wtGZ but not in AMEF#6101, of a larger or conformationally different epitope with a higher affinity for the ZL.2.2 paratope. Alternatively, anyhow, it could be due to the conversion, upon antibody binding to AMEF#6101, of a portion of the binding energy into the conformational change leading to the increased enzymatic activity of this otherwise defective betaGal protein.

These three monoclonal antibodies were able to activate, beyond AMEF#6101, also other AMEFs, differing, however, both in the extent of their activation potency and in the preference for single AMEFs. As shown in Table 4, ZL.2-2 was very active on AMEF#959 and AMEF#918, ZL1-1b (definitely less potent) could instead induce a significant activation of AMEF#645 and AMEF#918 (but not of AMEF#959), while the preferred targets of ZL2.1b (that disdained AMEF#918) were AMEF#645 and AMEF#959.

In the same Portovenere meeting of October 1981, a new entry among the AMEF Labs was Julian Duncan's team from the Wellcome Research Laboratories in the U.K. Having generated hybridoma cell cultures through fusion of myeloma cells with splenic lymphocytes from HO rats or from Balb/c mice immunized against the wtGZ protein, they obtained[30,31] four different monoclonal antibodies that, upon addition to AMEF#13, caused a variable but significant extent, the basal enzymatic activity of this defective betaGal protein. One of them, BG79, was even capable of inducing further activation of AMEF#13 proteins that had already interacted with one of the other three monoclonals (Figure 11 and Table 5). The overall magnitude of these synergistic activation processes was often larger than the sum of the effects produced by the two collaborating antibodies.

The affinity of radioiodinated BG79 for polyvinyl plates coated with AMEF#13 was so strong that it could displace only by its unlabeled homologue, but not, to any extent, by an excess of any other monoclonal.[31]

These results indicate, according to Duncan's team, that the paratope of each monoclonal interacts with a different and independent epitope of the AMEF#13 protein, leading to distinct conformational effects that can result in a highly synergistic activation.

An accurate study of the pre-steady state of the activation process showed (Figure 12) that the time course of AMEF#13 activation upon addition of monoclonal antibody BG.79 had a $t_{1/2}$ value (at 30°C) of 4.2 minutes (corresponding to an association rate with a value of 0.17 + 0.01 min^{-1}). If the same AMEF had been partially pre-activated by pre-incubation with monoclonal BG.81, the acquisition of

Labeled monoclonals	% displacement by unlabeled antibodies from hybridoma clones		
	Unlabeled ZL.1-1b	Unlabeled ZL.2-1b	Unlabeled ZL.2-2
^{3}H-ZL.1-1b	**−71.9%**	−7.8%	−93.8%
^{3}H-ZL.2-1b	−14.1%	**−75.0%**	−70.3%
^{3}H-ZL.2-2	−68.0%	−40.6%	**−75.0%**

TABLE 3 Competition, in terms of binding capacity to polyvinyl-adsorbed wtGZ, between monoclonal antibodies from different anti-wtGZ hybridoma clones

Note: Monoclonal antibodies were biosynthetically labeled by addition of ^{3}H-leucine to the corresponding hybridoma cultures. Comparable ^{3}H-labeled aliquots of the culture fluids from these hybridomas were added to the wells of a polyvinyl plate pre-treated with wtGZ, and allowed to react with the adsorbed GZ in the presence (or absence) of saturating amounts of homologous or nonhomologous unlabeled culture fluids. The % displacement of radioactivity by the unlabeled antibodies is expressed as % of the value obtained in the absence of any competing antibody. The displacement values occurring upon addition of homologous culture fluids are shaded. The bold values indicate, for each labeled monoclonal, the maximum displacement that can be obtained by using the same unlabeled monoclonal.
Source: Data from fig. 2 by Accolla et al.[28]

TABLE 4 Capacity of monoclonal antibodies present in the fluids of 3 hybridoma clones to activate different AMEFs

Hybridoma clones	% increase of AMEF activation (over the basal levels of enzymatic activity of each AMEF)				
	#627	#645	#6101	#959	#918
ZL.1-1b	+20%	+80%	+90%	+20%	+80%
ZL.2-1b	+10%	+70%	+360%	+ +220%	+10%
ZL.2-2	+10%	+20%	+ +1440%	+ +590%	+ +510%

Source: Modified from table 1 by Accolla et al.[29]

the further synergistic activation occurred with a $t_{1/2}$ value at 30°C of 5.1 minute, that is, with a significantly lower value (namely 0.12 ± 0.01 min^{-1}) of the association rate.

14 | ACTIVATING VERSUS INACTIVATING anti-wtGZ ANTIBODIES: MODULATION OF BETAGAL IMMUNOGENICITY

An intriguing observation during the first AMEF experiments was the high variability in the activating titer of anti-wtGZ antibodies. The best results were obtained with immune sera from New Zealand rabbits, while many experiments performed with antibodies obtained from immunized mice never reached the activation level of rat or rabbit antibodies (Strom and Celada, unpublished results). In their investigations of the activation process involving AMEFs generated by a variety of _E coli_ strains characterized by different missense point mutations in their lacZ gene, Melchers and Messer had verified[19] the possibility of a selective adsorption of their activating anti-wtGZ antibodies (elicited in rabbits) on two different Sepharose-bound AMEF proteins. The various fractions eluted from the Sepharose column had been found to possess a non-uniform ability of activating a same AMEF; this result was interpreted as indicating either a heterogeneity within the population of activating antibodies, or their co-existence with other "activation-inhibiting" antibodies.

In order to examine in detail the functional diversity of their anti-wtGZ antibodies elicited in SJL/J mice, Frackelton and Rotman[32]

obtained sets of hybridoma cultures by hybridizing spleen cells from immunized mice with NS-1 myeloma. They examined then the effects of the supernatants of 30 hybridoma microcultures in terms of their ability to bind wtGZ, to protect it from heat inactivation, and/or to induce AMEF activation or, viceversa, wtGZ inactivation. Out of 33 supernatants from hybridoma subclones that contained anti-wtGZ antibodies, 12 of them had no other effect, 8 caused inactivation of the wt enzyme, and 13 were able to promote the latent enzymatic activity of #6101AMEF. None of them possessed at the same time, AMEF-activating and wtGZ-inactivating capacities (unfortunately no assay was performed to verify what happened to the latent enzymatic activity of #6101AMEF when the AMEF-activating and wtGZ-inactivating antibodies were allowed to act simultaneously).

A detailed study of the kinetics of antibody production in mice that had been immunized with wtGZ was performed by Kunkl et al,[33] with the aim of verifying the possible heterogeneity, in terms of their effect(s) on #918AMEF activation, of these antibodies. There was, soon after the secondary challenge, an early rise of the AMEF-activating antibodies, but their titer declined at later times. A similar transient production of activating antibodies was observed in vitro when the supernatants of co-cultures of GZ-primed B cells and GZ-primed T cells were tested, by using an in vitro helper assay, for their ability to induce AMEF-activation. When these supernatants were adsorbed on denatured (reduced and carboxymethylated) GZ-protein, it could be shown that high levels of activating antibodies were, in fact, present also at longer times, but could be detected only after removal of some "inhibitory" antibodies directed against some
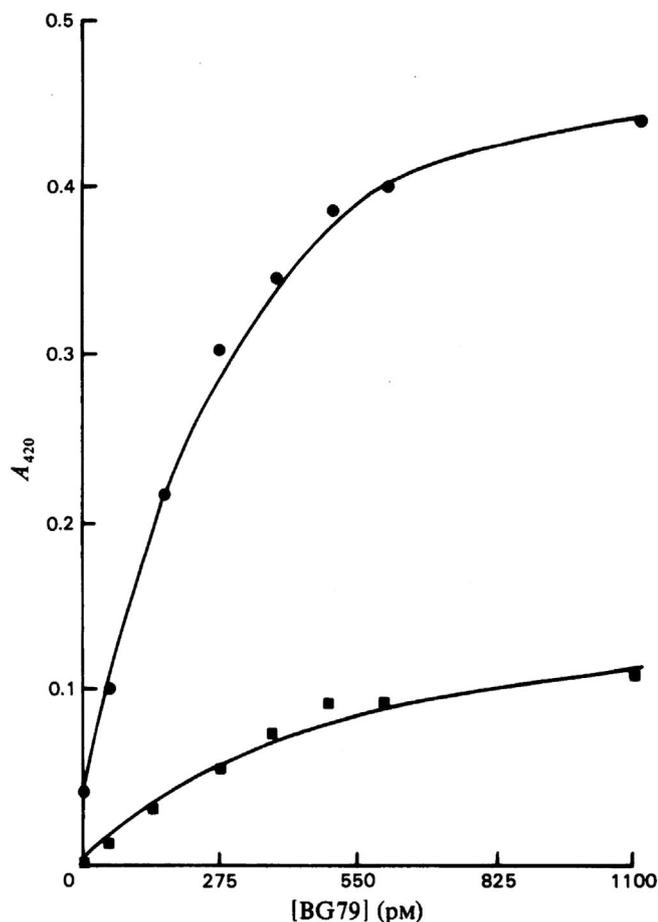
FIGURE 11 Synergistic activation of AMEF#13 by BG79 and BG81. AMEF#13 was allowed to interact for 3 hours with various concentrations of BG.79 in the presence (full squares) o absence (full circles) of 10 pmol of BG.81. The enzyme activity of the activated AMEF was then evaluated by measuring the absorbance at 420 nm of the nitrophenyl moieties produced by ONPG hydrolysis (fig. 1 by Duncan et al[30])

TABLE 5 Synergistic activation of AMEF#13 defective betaGal monoclonal antibodies produced by anti-GZ hybridoma clones

	Enzymatic activity (units/min at 37*C)			
	First monoclonal added			
	BG.79	BG.18	BG.19	BG.81
With no other addition	7.6 ± 0.4	12.0 ± 0.3	1.0 ± 0.1	0.86 ± 0.04
Upon addition of BG.79	—	42 ± 1	11.0 ± 0.2	28 ± 0.1

Source: Modified from table 2 by Duncan et al.[30]

sequential determinants of the denatured GZ protein. Among hybridoma microcultures whose supernatant was able to bind the denatured GZ, only 8% of them were capable of inhibiting AMEF activation - but no assay was made to verify whether they could also suppress the enzymatic activity of wtGZ.
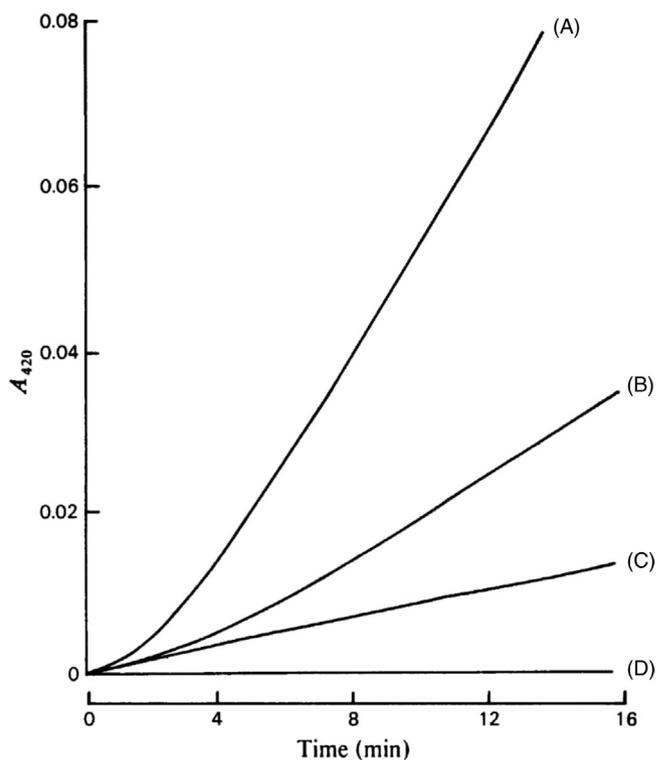
FIGURE 12 Pre-steady state of activation by monoclonal antibodies. Lines B and D are the best-fit of the results obtained (in 19 experiments) by adding at time 0 the betaGal substrate ONPG to a solution (kept at 30°C) containing 1.5 pmol of AMEF#13 that had been preincubated for 2 hours in the presence (curve B) or absence of BG.81. Lines A and C are the best fit of the results obtained (in 10 experiments) by adding at tine 0, to the same AMEF#13 solutions that had been preincubated in the presence (curve A) or absence (curve C) of BG.81, the betaGal substrate ONPG together with BG.79. The ordinate indicates the time course of o-NP release by the activated betaGal (fig. 2 by Duncan et al[30])

Kunkl et al indicated two different mechanisms as potentially responsible of the late wave of inactivating antibodies: a) the proteolytic degradation of the antigen by intra or extracellular enzymes - thus inducing generation of antibodies against sequential determinants after termination of the initial immune response to conformational epitopes; b) an involvement of macrophages in binding and partially degrading, through a Fc-receptor pathway, antigen-antibody complexes formed in the initial immune response – thus initiating a very efficient major histocompatibility complex-restricted presentation to T cells and stimulating B cells to generate antibodies against some epitopes that were cryptic in the native macromolecular antigen.

15 | STRUCTURAL INTERPRETATION OF THE MECHANISM(S) INVOLVED IN AMEF AND SECOND WAVE RESULTS

The *lacZ* gene of wild-type *E coli* is encoded a protein sequence of 1023 amino acid residues that spontaneously associates into dimers that are enzymatically inactive and then into a tetrameric

enzyme with high β-galactosidase activity. In each protomer, the first 50 residues have a "quasi-random" extended structure, named "α-complementation peptide" for its ability to restore the enzymatic activity in defective proteins produced by some *E coli* mutants carrying a deletion of approximately the first 8% of the β-galactosidase structural gene. After this α-complementation peptide, the 3-dimensional structure of the remaining amino acid sequence of each protomer could be subdivided in five domains[34]:

1. Domain 1 (aa. 51-to-219), characterized by a jelly-roll like extended structure;
2. Domain 2 (aa. 220-to-334), structurally similar to a fibronectin type III barrel formed by a series of 7 β-strands, except for a so-called "protruding loop" (aa.277-to-288) that has a "disordered" random coil conformation;
3. Domain 3 (aa. 335-to-627) is a "TIM barrel"-like structure formed by a bundle of eight (α/β) elements, involved in the catalytic activity of the enzyme.
4. Domain 4 (aa. 628-to-736) is, like domain 2, structurally similar to a fibronectin type III barrel;
5. Domain 5 (aa. 737-to-1023) is essentially a 19 β-stranded sandwich.

The enzymatic activity of tetrameric β-galactosidase requires that the subunits of two different dimers associate through so-called "activating interfaces," where the α13 and α14 helices of the third and fourth (α/β) elements of each subunit face the corresponding helices of another subunit of a different dimer (Figure 13).

The activating effect is mediated by two particular details of this association:

1. insertion of a portion of the "alpha-complementation peptide" into a "tunnel" between domain 1 and domain 3, so as to allow positioning of the peptide extremity near some amino acid residues of domain 3 that are located near the very short helix (α15) of its 5th (α/β) element;
2. interaction of the apex (Glu281) of the "protruding loop" of domain 2 with the region (residues 510-to-519, among which in particular Ala514) that precedes, in the amino acid sequence, the α15 helix of the 5th (α/β) element of domain 3 of the opposite subunit.

It is obviously impossible to fulfill the former requirement in the case of a deletion involving the operator-proximal segment of the betaGal gene - but activity can then be restored by addition of an α-complementing peptide. Any disturbance of the domain 3 region involved in the activating interface (that includes helices α13, α14, or the 501-514 sequence adjacent to the abortive α15 helix) is also likely to be responsible of the decreased enzymatic activity exhibited by defective betaGal proteins encoded by a *lac*Z gene affected by a missense point mutation. How could then the interaction with an anti-
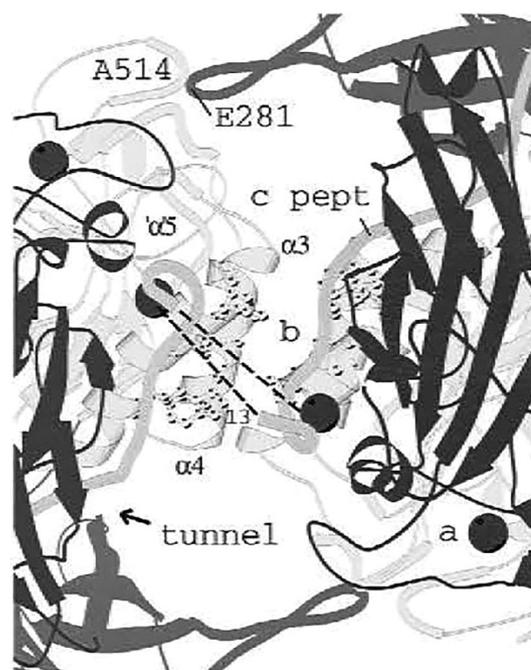
FIGURE 13 Activating interface of two β-galactosidase subunits in the tetrameric enzyme, in the proximity of the four-helix bundle and of the complementation peptide. The activating association between two antiparallel subunits is ensured by a four-helix bundle participated by the α3 and α4 helices of the (α/β)₈ barrels of each subunit. The interface contains also the "protruding loop" (identified by Glu281) that extends across the interface toward an "α5" position of the opposite subunit, corresponding to the short ("missing") α5 helix of the (α/β)₈ barrel. The complementation peptide, labeled "c pept." starts at residue Arg13 [near the letter b] and extends through a tunnel made by Domains 1 and 3 (from fig. 6C by Juers et al[35])

wtGZ antibody be able of correcting such a "disturbance"? To this purpose, the above-mentioned detailed structural knowledge of the betaGal protein (wild type or AMEF) needs to be combined with an adequate investigation of the possible paratope(s) of an antibody specifically activating a given AMEF.

A real turning point in this direction was accomplished in 1998 (30 years after Rotman-Celada's first description of AMEF) by Martineau et al,[36] who succeeded in transferring the gene of a monovalent fragment of an anti-GZ antibody into *lac*⁻ *E coli* cells (selected for having a particular AMEF gene). It became possible, by selecting the bacterial cells that had acquired the ability of fermenting lactose, to obtain large amounts of the antibody fragment that could specifically activate the AMEF present in those bacteria. *E coli*, where the biosynthesis of that antibody fragment was transferred, offers the advantage of a large variety of mutant strains, but also of a detailed knowledge of its genetics and its biosynthetic pathways. Martineau's team[37] could thus show that the same scFv13 antibody fragment possessed the ability of activating three different AMEFs, namely #645, #6101, and #959.

Large amounts of the antigen-antibody complex were on the other hand made available by this bacterial system. This allowed a

very accurate crystallographic study, published in 2014 by Vinothkumar et al[38] that verified the contacts between an scFv13R4 fragment and AMEF#959 (known to carry a Glu359 -> Lys mutation). A detail of how the AMEF#359-scFv13R4 adduct can be reconstructed on the basis of that study is shown in Figure 14.
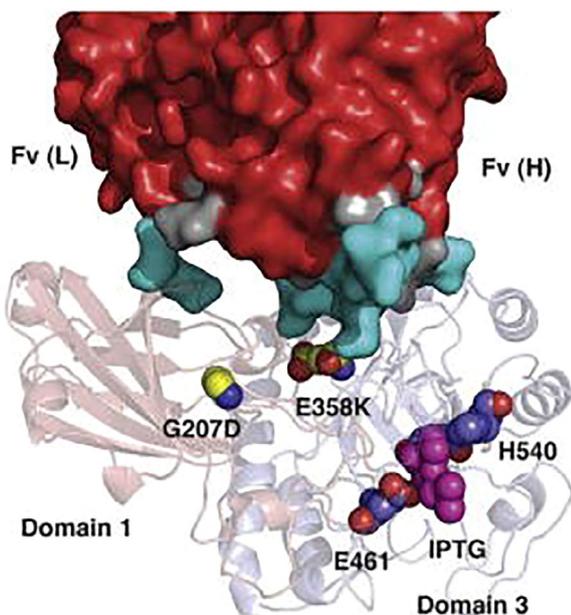
FIGURE 14 Reconstruction of the AMEF#959-scFv13R4 antigen-antibody complex (Partial reproduction of fig. SI.4 from Supplemental Info by Vinothkumar et al[38]). IPTG is a betaGal substrate analog, positioned between residues Glu461 and His540 of the substrate-binding site of betaGal. E358 is the position of the mutated residue of AMEF#959, G207 shows the position that would have the mutated residue of AMEF#645. The cyan-colored region indicates the region of AMEF#959 directly involved in the contact with the red-colored scFv13R4 antibody fragment [whose light and heavy chains are indicated by Fv(L) and Fv(H)]

A first analysis of antigen-antibody interactions is often performed[39] by using strictly "sequential" (or "continuous") criteria to identify epitopes contacts with some corresponding paratopes. Since the Glu -> Lys mutation affecting residue 358 of AMEF#959 is probably responsible for the loss of basal activity of this defective betaGal protein, attention was focused on the contacts of some adjacent betaGal sequence (notably the Pro361-His370 continuous epitope) with some paratope of the *scFv13R4* "activating" antibody fragment. As shown in section *b* of Table 6, the above-mentioned sequential epitope of AMEF#959 is indeed in very close contact with a paratope formed by Glu 31, Ser 33, Ser 53, Ser95, and Ser96 from the heavy chain of the scFv12R4 antibody fragment.

As shown in section *d* of Table 6, however, the Ser33 and Ser 53 components of this paratope are, however, in contact, together with Ser54, also with the Ala609-Thr612 sequence of the betaGal antigen that would constitute another continuous epitope of the same protein. Due to their common interaction with the Ser33 and Ser53 residues of the same paratope, these two continuous epitopes (namely Pro361-His370 and Ala609-Thr612) are likely to have adjacent positions in the three-dimensional structure of the betaGal domain 3, and can, therefore, be considered[39] as two components of a same "conformational" (or "discontinuous") epitope. A proximity of His357 to Phe601 had been indeed confirmed by Juers et al[40] in their three-dimensional reconstruction.

In our opinion, however, this simplistic conclusion of a correcting paratope acting through a direct interaction with (or very near to) the mutated amino acid (residue 358 in the case of AMEF#959) can hardly be generalized in order to account for the ability of the same scFv13R4 antibody fragment[36,37] to "activate" also AMEF#645 and AMEF#6101, whose point mutations are in the first or second domain. We can hardly imagine that these other point mutations can find, in the same scFv13R4 antibody fragment, other paratopes able to interact specifically with their altered sequences.

TABLE 6 Residues involved in actual contacts of AMEF#959 with scFv13R4

Continuous epitopes of AMEF#959 (amino acid residues involved in the contact with the antibody)	Secondary structure present in the corresponding region of wt-betaGal	Antibody residues involved in the contact with AMEF#959 (possible "activating paratopes")
(a) Asn 55 and Glu 57 (in betaGal domain 1)	Random coil region linking 2 short extended β stretches	Ser 56 of the CDR2 region of the L chain and N-terminal Glu1 of the H chain
(b) Pro361, Leu362, His363, and Gln370 (in the pre-catalytic portion of betaGal domain 3)	Random coil region that immediately precedes the α11 helix	Glu31 and Ser 33 of the CDR1 region of the H chain + Ser53 of the CDR2 region of the H chain + Ser95 and Ser96 of the CDR3 region of the H chain
(c) Ile576, Lys 577, Trp578, Asp579, Glu580, Gln581, Gly582, Gln583, and Pro584 (in the catalytic portion of betaGal domain 3)	Random coil region followed by a short extended β stretch	Ser30 of the CDR1 region of the H chain + Arg71, Gln73, and Ala74 of the external loop of the H chain
(d) Ala609, Asp610, and Thr612 (in the catalytic portion of betaGal domain 3)	End of an extended β stretch, followed by a random coil region	Ser33 of the CDR1 region of the H chain + Ser53 and Ser 54 of the CDR2 region of the H chain

Source: From table 4 by Vinothkumar et al,[38] modified.

The same problem occurs also with classical monoclonal antibodies produced by hybridomas. The three different monoclonals obtained by Accolla et al[28,29] bind to adjacent sites of AMEF#6101, but exhibit considerable differences in their activating ability of this defective enzyme - giving a rather typical example of the complexity of the interaction[41] between "fuzzy" epitopes and discontinuous paratopes. One of them, ZL2.2, which induces a 15-fold activation of AMEF#6101 (mutated in the distal region of domain 2), is also able to increase by over 500%, the enzymatic activity of AMEF #959 (mutated in residue 358, located in the initial portion of domain 3), and even of AMEF#918 (whose mutation is reportedly in domain 5). A more restricted, but still present, multiplicity of targets is also possessed by monoclonals ZL1.1b (which is unable to activate AMEF#959) and ZL2.1b.

The various monoclonals isolated by Duncan's team[30,31] support the idea that their paratopes can exert their activating effects on the same AMEF#13 protein by interacting with distinct, non-competing epitopes. Their activating abilities were not only able to sum to each other, but proved to be synergistic - suggesting that the binding of a single monoclonal is causing, within a single betaGal macromolecule, a conformational modification not only of its target but also of some nearby region, thus amplifying the effect of another monoclonal.

The previously described structure of β-galactosidase (and of other betaGal-related proteins) indicates that the so-called "activating interfaces" of the different protomers are the regions that exert a control on subunits association into a tetramer as well as on the enzymatic activity. Each interface is located in a central region of domain 3, and contains two right-handed helices (α13 and α14), members of the third and fourth (α/β) elements of the "Triosephosphate IsoMerase (TIM) barrel"-like structure of domain 3. As previously shown in Figure 13, this interface is flanked by a random coil sequence (residues Asp510-to-Ala514) able to bind the "protruding loop" (residues Gly277-to-Ala288) from domain 2, and contains also the very short α15 helix, which is the point of contact with the extremity of the α-complementation peptide.

Although most enzymatically defective AMEFs have been shown to have a tetrameric structure despite their low enzymatic activity, the easy dissociation of AMEF#6101 into a dimeric state after a short exposure to 37°C suggests a certain fragility of the activating interfaces of their subunits - fragility that is overcome by the interaction with the activating antibodies. No paratope can, however, take any direct contact with these quasi-planar regions of the activating interfaces, which are locked by their unique role of mediating inter-subunit association.

By analyzing the three-dimensional organization of the betaGal protomers (as described by Jacobson et al[34]) at the light of the primary and secondary structures, also the first two α-helices of domain 3, namely α11 and α12, appear to be positioned in a quasi-planar layer that lies just below the activating interface. The α11 helix (that starts at Gln370), is preceded, in the amino acids sequence, by a random coil segment (Asn355-to-Glu369), whose components interact (according to Juers et al[35]) with the lateral chains of some residues from the distal portion of domain 1 (eg, His357 with Asp201). On the other hand,

also the terminal portion of domain 2 (from Leu310 to Asp331) has a planar sheet conformation with two typical β-stretches. Just below the critical "activating interface" of domain 3, we have, therefore, two planar or quasi-planar layers: a lowest one formed by the β18 and β19 stretches of domain 2 and an intermediate one that contains the α11 and α12 of domain 3.

Gln358, whose substitution by Lys358 is the result of the missense point mutation encoded in the lacZ gene of AMEF#959, is indeed one of the amino acid residues of the above-mentioned random coil sequence that precedes the α11 helix. This sequence contains those residues (Pro361, Leu 362, His363, and Gln370) that, as shown in Table 6, form the continuous epitope, which is the target of the presumed activating paratope of the scFv13R4 antibody fragment. The epitope-paratope interaction is likely to cause, in the architecture of this region, a distortion that can be easily transferred to the "activating interface" that lies immediately above it. Such a multistep process is clearly in line with the observed first-order character of antibody-mediated AMEF activation reactions.

This mechanism, which has been suggested by the structural features of AMEF#959 and by its interaction with a paratope of scFv13R4, can be easily extended to all the other AMEFs characterized by mutated residues upstream of residue 358. Since an antibody raised against a specific AMEF is unable to activate its own antigen, the various AMEFs can, as shown in Table 7, be divided in subgroups (**a, b, c**) according to the cross-activating ability by antibodies raised against other members of the same subgroup. We can presume, anyhow, that a population of anti-wtGZ polyclonal antibodies contains a variety of activating paratopes, capable of generating, through distinct interactions with nearby epitopes of an AMEF protein, a series of small conformational changes converging on the activating interface. This overall activating process of an AMEF can however be antagonized, as reported by Celada et al,[8] by antibodies against that particular AMEF or by events able to interfere with the required intrinsic flexibility of the betaGal protein.

In addition, the observed potentiation and acceleration effect exerted by an anti-wtGZ polyclonal antibody on the *del*M15 protein in the presence of a limited amount of α-complementation peptide can be accounted for by a similar mechanism - that can even produce a very limited (and in fact abortive) enzymatic activation of the *del*M15 protein alone.

As compared to the fast ($t_{1/2}$ < 5 min) activation of AMEF#13 by Duncan's monoclonal antibody BG.81, polyclonal antibodies need somewhat longer times in order to exert their activating effects: under similar conditions, the $t_{1/2}$ values for activation were of 12 minutes[19] for AMEF#645 (which belongs to the same subgroup *b* of Table 7), and of approximately 40 minutes[8] for AMEF#6101 (subgroup *c*). These different values of $t_{1/2}$ - and of the activation rates - can depend on a different complexity of the activation process or on different properties of the AMEF subgroups.

The few experimental data on the two AMEFs (#40 and #919), whose mutated residues are in domain 5 of the betaGal subunits (and have been, therefore, classified as belonging to the so-called MMgroup2 and to subgroup d of Table 7) do not allow a comparable

TABLE 7 Structural interpretation of the mechanisms(s) involved in antibody-mediated enzyme formation (AMEF)

AMEFs	Structural parameters			Enhancing effect, by specific antibodies, on betaGal enzymatic activity						
	Protomers assembly	Domain and amino acid(s) affected	Secondary structures near the missense mutations	Anti-GZ	Anti-Group 1	Anti-#6101	Anti-#X7	Anti-Group 2	Anti-Ω-donor	scFv fragment
(a) #627	Tetramer	Domain 1, distal portion; region from aa.171 to 176	β/α barrel (β stretch + α helix)	YES						
(b1) #13 and other AMEFs of Group 1MM (#71, #429, #630, #779, 950)	Tetramers	Domain 1, distal portion, region from aa. 188 to 200	two adjacent β stretches	YES	NO	YES	YES	YES ?		
(b2) #645	Tetramer	Domain 1, distal portion; aa.207 [Gly207Asp]	End of α β stretch	YES	NO	YES	YES	YES?		YES
(c1) #6101	Dimer (?) > Tetramer	Domain 2, distal portion, region from aa. 298 to 330	Two adjacent β stretches	YES	NO	NO	YES	YES	YES	YES
(c2) #X7	Tetramer	Domain 2, distal portion, at or near aa. 328	β stretch	YES	NO	NO		YES	YES	
(c3) #959	Tetramer	Domain 3, proximal region; aa. 358 [Glu358Lys]	End of α β stretch	YES	NO					YES
ΔM15 (α-acceptor)	Dimer	Deletion of aa.11-41		YES (partial)						
ΔM15 + some α-peptide	Tetramer	Result of partial α-complementation		YES						
ΔM15 + excess α-peptide	Tetramer	Result of extensive α-complementation		NO						
(d) #918 and #40 (Group 2MM)	Tetramer	Domain 5; region from aa. 883 to 919	three adjacent β stretches	YES	??(+/−)	YES	NO	NO	YES	
Ω-acceptor (alone)	Tetramer	Deletion of domain 5		NO		NO	NO	NO		
Ω-acceptor + some Ω-donor	Tetramer	Result of partial Ω-complementation		YES		NO	NO	NO		
Ω-acceptor + excess Ω-donor	Tetramer	Result of extensive Ω-complementation		NO			YES	YES		

Source: From table 3 by Celada and Strom,[26] modified.

evaluation of the reasons of their low enzymatic activity or of the steps that are likely to be involved in their antibody-mediated activation process.

It is, however, reported, in the detailed study by Juers et al,[40] that residue Trp999 of domain 5 is, together with residues Arg599, Phe601, Gly794, and Ser796 of domain 3, a member of a three-dimensional region that exerts a strong control of the rate of release of the products of the β-galactosidase reaction. This very critical effect is due to changes in orientation and/or in conformation (from an "open" to a "closed" state) of the boundary region between the distal portion of domain 3 and domain 5. The partial amputation of this boundary region leads, in the truncated Ω-acceptor protein from *E coli* strain S9080, to a loss of enzymatic activity that can be restored by addition of the Ω-donor from strain W4680 (and even better when this Ω-donor is interacting with anti-wtGZ antibodies). A distortion of this same region can also be linked, in AMEFs #40 and #918, to a low basal enzymatic activity, susceptible of being enhanced by interaction with some paratope of the anti-wtGZ antibodies (or of antibodies against AMEFs of MMgroup1) and also by antibodies elicited against an Ω-donor.

We have instead no indication about the mechanism involved in the activating interaction exerted by anti-Ω-donor antibodies on AMEFs #6101 and #X7. We can only notice that, according to Juers et al,[40] the Phe601 residue involved in the interdomain boundary region is a member, together with Asp201, His357, and Trp568, of another key region that, upon binding of the substrate to the enzymatically active β-galactosidase, shifts the enzyme-substrate complex from a "shallow mode" to a "deep mode" structure. Phe601 is also rather close, in the primary betaGal sequence, to the Ala609-Thr612 continuous paratope involved, as reported in Table 6, in the binding of scFv13R4 fragment to AMEF#959.

16 | CONCLUDING REMARKS: A NEW FUNCTION FOR ANTIBODIES?

The activation of enzymatically defective betaGal proteins by antibodies and by their Fc-deprived Fab fragments can be considered a relatively new facet within the arsenal of the adaptive immune system (AIS). This statement can be better defined and qualified by considering that betaGal proteins and Antibodies are two groups of macromolecules synthesized by organisms belonging to very distant evolutionary Eras, two and a half billion (2 500 000 000) years apart. The "Elder" partner of the "Rhode Island 1967" experiment was a "defective" version of a bacterial galactosidase that had lost most of its enzymatic activity because of "one strike from the stars" that had modified just one coding unit of the GZ structural gene, and therefore, changed a single amino acid of the protein sequence. The ternary and quaternary structure of the mutated protein is likely to be similar, but not totally identical, to the original β-galactosidase - local differences being also present in some of its sequential or conformational "epitopes," *i.e.* in clusters of adjacent amino acid residues that, being exposed on the surface of the antigenic protein, are available for

interaction with appropriate antibodies. The "Younger" partner was instead an Antibody synthesized by a mammalian organism that, during its incredibly long evolution process, had developed, in the framework of an AIS suitable to perform a defensive program against invaders, a population of B-lymphocytes able to recognize, through their membrane receptors, the epitopes from any "non-self" antigen. Cell proliferation cycles, combined with hypermutation events, will thereafter lead to selection and multiplication of the antibody-producing B-cell clones having the highest specificity for the epitopes of a given antigen. Some amino acid clusters (called "paratopes") of the N-terminal (antigen-binding) regions of the antibodies produced by these B-lymphocytes will then be so high that each antibody binding site will appear to be almost a "negative image" of its antigenic target.

According to the abduction of the actual experimental design that led to AMEF discovery, if a mutation had slightly affected the shape of some epitope(s) of an enzyme protein (causing a loss of the enzymatic activity), the antibody paratopes specific for the pristine enzyme would either refuse to recognize the altered epitopes or, in the presence of a sufficient degree of compatibility between their three-dimensional architecture and the altered epitope structures, the minimal free energy value within the paratope-epitope adducts would be restored by a mutual conformational adjustment and reactivation of the defective catalytic activity.

A positive result was not only obtained in the "Rhode Island 1967" experiment with the W6101 mutant strain, but was also confirmed and extended in the following studies. It can be attributed to the fact that the betaGal proteins, as well as the anti-wtGZ antibodies, have complex quaternary structures that, especially upon mutual interaction and formation of adducts, can reasonably have a variety of possible conformations with intermediate levels of free energy minima. The "reconfirming" effect exerted by some anti-wtGZ paratopes on AMEF proteins characterized by a conformation slightly different from the wtGZ enzyme is rather similar to the so-called "allosteric regulation" of several proteins involved in multimeric structure or in the transmission of signals to other protein components. In the AMEF experiments, however, the role of allosteric regulator of some bacterial proteins is played by the paratopes of some antibodies (or of their Fab fragments) that have been generated on purpose as TOOLS (planned by human experimenters and addressed to specific epitopes).

In classical immunology, immune complex formation is known to lead to highly relevant conformational effects, revealed, for example, by the increased affinity of the Fc receptor of antibodies for the first factors of the complement cascade - thus initiating most of the defense programs against Invaders. In the in vitro AMEF experiments, instead, most of the complexity of the AIS was, however, disregarded on purpose because there was no need for the activation a complete Defense Program, and the activating antibodies (that could, in fact, be replaced by their Fab fragments) were utilized as if they were just chemical reagents. As it happens in other recent developments of immunochemistry,[41] the epitope-paratope interactions appear to have surprising mimicring and pro-active properties. Some antibody paratopes appear, on the other hand, to be endowed with an intrinsic

high degree of "flexibility."[42] The "immune attraction" can be high enough to generate not only a tight interaction between a given epitope and the chosen paratope(s) borne by antibody fragments, but also to be responsible for the energy which is needed to impose a change of local equilibria. The interaction between the epitope(s) of the various AMEFs and the paratopes of anti-wtGZ antibodies cannot, however, be viewed as linking complementary structures within a rigid environment, similar to that formed by commercial "LEGO bricks", but as a complex mechanism that involves relatively flexible macromolecules endowed with a high conformational energy that allows a variety of potential structures.

Many monoclonal antibodies are at present used for therapeutic purposes, especially in oncology. Most of them have been raised against some pathologic membrane proteins involved in generation or transmission of signals that activate cell proliferation, and act essentially by binding to some particular region of their target, which is thus prevented from performing its pathogenic task. The AMEF experiments suggest that antibodies against inactive variants of these membrane proteins might also be able to achieve some therapeutic effects by inducing a conformational change leading to the loss of pathogenicity. Martineau's team has shown[43] that the procedure that they had developed for the production, by appropriately modified *E coli* cultures, of large amounts of specific AMEF-activating antibody fragments can be also extended to antibody fragments specific for other protein sequences. These fragments, whose small size would ensure easy penetration in almost any body compartment, would be expected to exert, as compared to the classical "blocking" monoclonal antibodies, more subtle conformational effects on their protein targets. We hope that sooner or later some step will be done in this direction.

The Piano. This final section of the Historical Review is reminiscent of a piano duo piece played with many cross-hand notes.

As you see, a number of publications belonging to the "Second Wave" contributed significantly to AMEF's legacy.

The strategy of the specific immune defense is based on a multiplicity of solutions: the Paratope (a 3-D negative picture of an Epitope), the tagging of the invaders, and the stored memory, its ever growing knowledge of the surrounding microscopic world.

However, its utilization, in June 1967, at Brown University was neither immunological nor defensive, but an experiment credited to two scientists, who borrowed it from an anti-betagal paratope. Luckily, the Paratope even exceeded its contracted duties: it found the target and began to exert its influences on the local conformation.

Human Intelligence has contributed to the AIS from the times of the vaccine, when it understood that it could propose to AIS any target at any time so that it could become fast in producing the antigen, when needed.

The best example is the invention of the RNA vaccine that is saving lives in the present Covid pandemics. This vaccine is indeed a combined application of genetic and immunological knowledge, enhancing the activity and speed of AIS.

Happy Tale. The Paratope managed to bind to the deranged Epitope, even with low affinity, thus allowing the betaGal's four levels of conformation to find the best shape, *i.e*, the original wt structure (as expected), including the immediate recovery of Enzyme function.

Discriminant. Although the mere use of a single Paratope, instead of the full antibody, appears to exert the required conformational effect, thus disproving a real immunological motivation, we have to consider that a monovalent binding is already known to exert a killing effect on a virus floating freely in body fluids. Anyway, the new function party was happy with the human-decided application that, as in the case of the AMEF team, decided the target.

Vicious. Is inactivation of a wt enzyme upon exposure to anti-AMEF antibody always due to a conformational effect? Not necessarily, it depends on the human motivation. The borrowed paratopes are absolutely innocent: they always follow the 2nd law of thermodynamics, and this is diriment.

Virtuous. For the cells in the body and in the lab, any act causing lowering of free energy is welcome, and promises calm and stability. The mark is always the "*wild*" type. To recover a deranged conformation is virtuous, a cure attempt will satisfy many equilibria.

New Wave and Hypothetical Heritage of Amef Legacy. Monoclonal antibodies, made available by the hybridoma technique, were incredibly advantageous in research and also in therapy. They are now improved and translated into a real industrial power. Facilitated brewing of FABs in *E coli* was another new invention that proved to facilitate their use, as it happened, for example, when AMEF activation was used as a signaling control for the cultures.[36]

Most in demand are monoclonal paratopes with capacities to hinder or cover contact points on cancer cells, a medical success that is still growing so far. But more advances are expected in the field of conformational modifications, reminding of the use on the mutant betaGal called AMEF.

A most promising regulatory protein appears to be the p53 tumor suppressor (TP53), a homotetrameric transcription factor very frequently mutated in human cancers. Its monomers have a modular structure, with an N-terminal portion that contains a transactivation domain, followed by a proline-rich region and then by a central core consisting of a large sequence-specific "DNA binding" domain. The C-terminal region contains an oligomerization domain followed by a disordered regulatory domain. As many as 29,000 somatic mutations of the p53 gene have been detected[44] in several human cancers. Almost 50 "small molecules" (among which at least 1 peptide) have been shown to possess the ability of "rescuing" – through a large variety of mechanisms[45]– mutated TP53 products, thus restoring at least partially, in some of them, the "correct" and/or "reactivated" conformation(s).[46] Similarly to what happened with AMEF, it can easily be abduced, in our opinion, that a conforming ability might also be possessed by some antibodies raised against native TP53 (or against single domains of this protein). By performing clonal selections of the corresponding antibody-producing cells, it should then be possible to obtain "reactivating" monoclonal antibodies, and then, by identifying their paratopes as well as the corresponding TP53 epitopes, clarify the multitudes of events connected to the TP53 correction mechanisms.

Further developments can also be expected on some prion-induced diseases, from Creuzfeldt-Jacob to Alzheimer's. These conformation-related paratopes should carry the negative image of the wt conformation of the prion, in order to reinforce, and even restore, wt conformation and restored function.

Total Cognition. One step further, stimulated by AIS's desire to "know" the invader in order to dominate or destroy it, is the following question: does the immune system have properties similar to those of a MIND?

The terms of "cognition" and "understanding" are used by us as metaphors. There re similarities between rational cognition and AIS in the extremely refined system of intercellular T-cell/B-cell cooperation, which is so clearly legitimized as part of the evolutionary protection of life.

Nature's "Mind". Van Regenmortel gladly admits, in a personal communication that metaphors are not the truth but often can be of help in facilitating understanding. Jacques Monod invented[47] the term "Teleonomy" to describe those phenomena of nature that seem to have a rational finalistic behavior but can be shown to be produced within the lines of evolutionary growth and selection.

Umberto Eco, a well-known Italian Semiologist, was impressed by the routine of AIS to avoid dangerous anti-self responses, where a B cell, before starting the release of antibodies, awaits the arrival of the helper cell, or dies. Eco declared the AIS acts as a "primitive unconscious cognitive system" and also found the AIS's routine similar to humans' response to an ambiguous message, i.e, by considering all possible meanings without doing anything until further information is received.[48]

Back to the Origin. The "Tree of Life" by the French biologist Lucien Cuénot[49]– reproduced in Figure 15 from an Italian book on Immunology by F.Celada– is a fitting reinforcement of two points made in these Concluding Remarks: first, to get a graphic impression of the two eras, from where the two contenders, betaGal and anti-body, originate, and, second, to allow the readers to verify the success of vertebrates in the transition from the Panocean to Dry Land. Note that on the right side the successful species without immunological
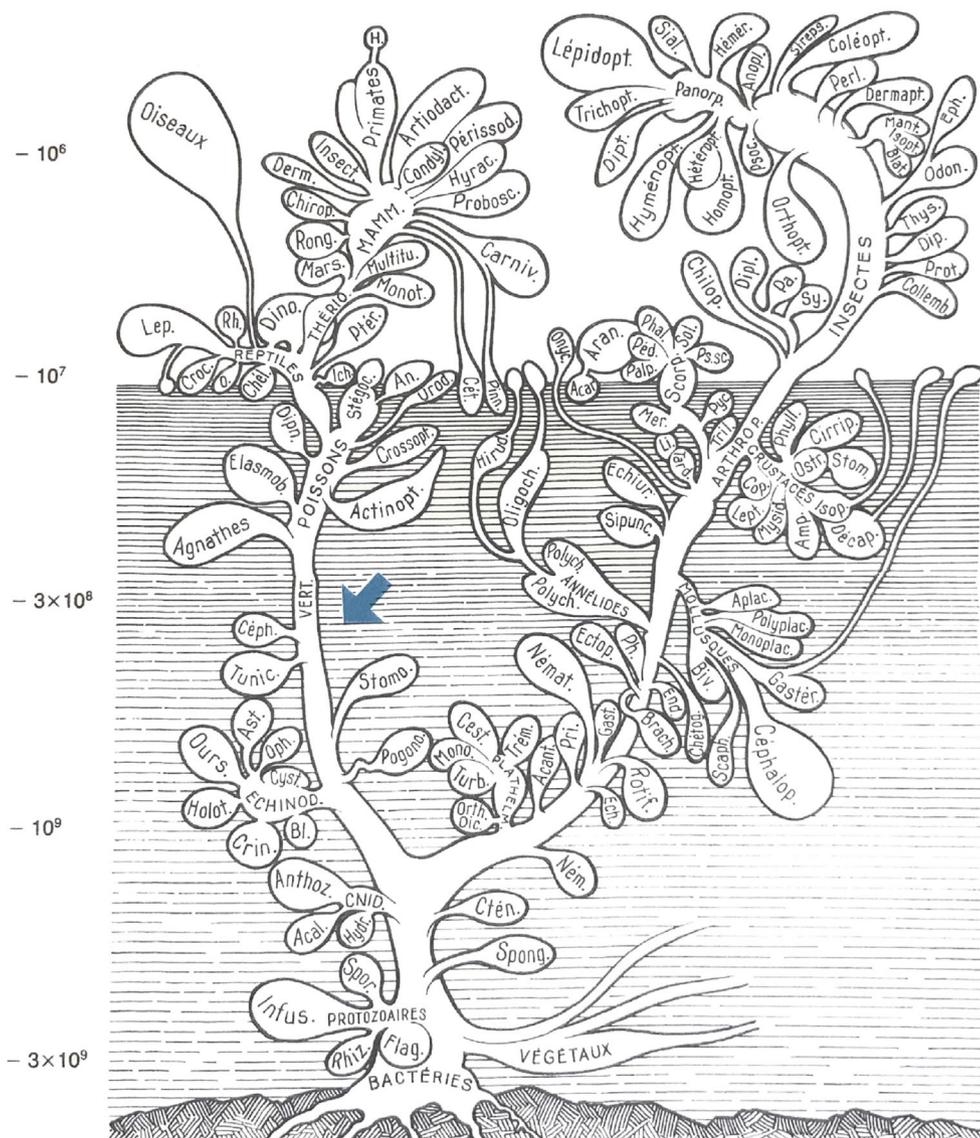
FIGURE 15 The Tree of Life, according to French biologist Lucien Cuénot (1866-1951), modified.[49] The beginning of life is set 3 billion years ago. The bifurcation between chordates and arthropods is set after 1 billion years. The added arrow in the left branch is pointing at 400 million years, and indicates the time of the last revolution of the adaptive, specific defense. Ten million years ago (-10^7), marks the end of the panocean

properties are huge in number but limited by their relatively recent appearance.

DATA AVAILABILITY STATEMENT

Being a historical review, no new data are presented in this paper.

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