Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 2036–2039, March 1989
Medical Sciences

Anti-idiotypic antibodies that protect cells against the action of diphtheria toxin

(diphtheria toxin receptor/toxin internalization)

JOHN M. ROLF*, HELEN M. GAUDIN*, STEPHEN M. TIRRELL†‡, A. BRUCE MACDONALD†, AND LEON EIDELS*§

*Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, TX 75235; and †Department of Microbiology, University of Massachusetts, Amherst, MA 01003

Communicated by Jonathan W. Uhr, December 16, 1988

ABSTRACT **An anti-idiotypic serum prepared against the combining site (idiotype) of specific anti-diphtheria toxoid antibodies was characterized with respect to its interaction with highly diphtheria toxin-sensitive Vero cells. Although the anti-idiotypic serum protected Vero cells against the cytotoxic action of diphtheria toxin, it did not prevent the binding of ^{125}I-labeled diphtheria toxin to the cells but did inhibit the internalization and degradation of ^{125}I-labeled toxin. This anti-idiotypic serum immunoprecipitated a cell-surface protein from radiolabeled Vero cells with an apparent M_r of ≈15,000. These results are consistent with the hypothesis that the anti-idiotypic serum contains antibodies that carry an internal image of an internalization site on the toxin and that a cell-surface protein involved in toxin internalization possesses a complementary site recognized by both the toxin and the anti-idiotypic antibodies.**

Diphtheria toxin (DT) is a protein of M_r 58,342 synthesized as a single polypeptide chain that can be proteolytically cleaved to yield two distinct fragments, the amino-terminal A fragment (M_r 21,167) and the carboxyl-terminal B fragment (M_r 37,195), which remain associated via a disulfide bond (1–4). The toxin possesses three functional domains: (*i*) the enzymatic domain, located in the A fragment, responsible for the NAD-dependent ADP-ribosylation of elongation factor 2 that results in inhibition of protein synthesis; (*ii*) the receptor-binding domain, located in the carboxyl-terminal region of the B fragment; and (*iii*) the internalization and entry domain, located in the middle of the B fragment (5, 6). DT enters cells via receptor-mediated endocytosis (7, 8) by first binding to specific cell-surface receptors followed by internalization of (toxin–receptor) complexes into endosomes and entry of the A fragment into the cytosol where that fragment inhibits protein synthesis. Cell-surface DT-binding proteins from highly toxin-sensitive Vero cells have been reported with apparent M_r values of 15,000–20,000 and have been proposed as the DT receptor (9, 10).

Anti-idiotypic antibodies raised against the binding sites (idiotypes) of antibodies to biologically important ligands have been used as cell-surface probes in such varied systems as the insulin receptor and retinol-binding protein receptor (11), the β-adrenergic receptor (12), the membrane receptor for factor H (13), the thyrotropin receptor (14), and a receptor for protein import into chloroplasts (15). Observations from these systems support the idea that some anti-idiotypic antibodies constitute an "internal image" of the original ligand and that a subset of these antibodies may recognize ligand-binding site(s) on cell-surface receptors.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Workers in one of our laboratories (S.M.T. and A.B.M., unpublished work) have isolated an anti-idiotypic serum prepared in inbred guinea pigs against anti-diphtheria toxoid antibodies (idiotype). The binding of the anti-idiotypic serum to the idiotype is specifically inhibited by diphtheria toxoid. Further, these anti-idiotypic antibodies could elicit an anti-anti-idiotypic serum that neutralized DT. These results prompted us to test the protective effect of the anti-idiotypic serum on the DT-mediated intoxication of Vero cells. In this communication, we report that this anti-idiotypic serum protects Vero cells against DT; however, the serum does so not by preventing binding of the toxin but by preventing its internalization. This anti-idiotypic serum recognizes a cell-surface protein with an apparent M_r of ≈15,000. These observations suggest that the cell-surface component recognized by the anti-idiotypic serum may be a physiologically relevant plasma membrane protein involved in internalization of the toxin. This protein may be the toxin receptor itself or another cell-surface protein.

MATERIALS AND METHODS

Materials. All chemicals used were of the highest purity available. All tissue culture reagents were obtained from GIBCO. Na^{125}I (IMS 30; 13–17 μCi/μg; 1 Ci = 37 GBq) and L-[4, 5-^{3}H]leucine (TRK 170; 64 Ci/mmol) were purchased from Amersham. Lactoperoxidase was obtained from Sigma and Pansorbin cells from Calbiochem. Partially purified DT was purchased from Connaught Laboratories (lot D525) and further purified by anion-exchange chromatography according to published methods (16) with modifications (9).

Antiserum. The preparation of the two anti-idiotypic sera employed will be published in detail elsewhere (S.M.T. and A.B.M., unpublished work). These two antisera were chosen from a large panel of anti-idiotypic sera generated by a variety of immunizing regimens as part of an ongoing study of guinea pig anti-idiotypic response. Briefly the procedures were as follows: *Protective anti-idiotypic serum.* Inbred (Hartley strain 13) guinea pig IgG1 specific for diphtheria toxoid (idiotype) coupled to keyhole limpet hemocyanin was used to generate an anti-idiotypic serum in inbred (strain 13) guinea pigs. This anti-idiotypic serum reacted with ^{125}I-labeled F(ab′)$_2$ fragments of anti-diphtheria toxoid but not with nonimmune guinea pig F(ab′)$_2$ fragments; this interaction could be inhibited by diphtheria toxoid but not by tetanus toxoid. Immunization of outbred guinea pigs with the F(ab′)$_2$

Abbreviations: DT, diphtheria toxin; αId, protective anti-idiotypic serum; NpS, nonprotective anti-idiotypic serum; PBS, phosphate-buffered saline; InsP_6, inositol hexaphosphate; IC_{50}, concentration of toxin that inhibits protein synthesis by 50%.

‡Present address: CIBA-CORNING Diagnostic Corporation, East Walpole, MA 02032.
§To whom reprint requests should be addressed.

fraction of this anti-idiotypic serum resulted in a serum (anti-anti-idiotypic) that neutralized DT in a rabbit subcutaneous toxicity assay. The anti-idiotypic serum, capable of eliciting neutralizing antibodies, will be referred to as αId. *Nonprotective anti-idiotypic serum.* This serum was generated in inbred (strain 13) guinea pigs immunized with diphtheria toxoid-specific guinea pig $F(ab')_2$ (idiotype) coupled to keyhole limpet hemocyanin. This anti-idiotypic serum reacted with ^{125}I-labeled $F(ab')_2$ fragments of anti-diphtheria toxoid, and this interaction was also specifically inhibited by diphtheria toxoid; however, the $F(ab')_2$ fraction of this anti-idiotypic serum did *not* elicit a neutralizing response when used to immunize guinea pigs. This anti-idiotypic serum will be referred to as NpS. In all experiments presented here, the characterized sera (αId or NpS) used were unfractionated.

Cell Culture. Vero cells (ATCC CCL 81) were maintained and passaged as described (9).

Cytotoxicity Assay. Tissue culture dishes (48-well) were seeded with 2.5×10^4 Vero cells and grown to confluency. Cells were placed on ice and washed several times with phosphate-buffered saline (PBS) containing $CaCl_2$ and $MgCl_2$ (PBS/$CaCl_2$/$MgCl_2$; 8.8 mM Na_2HPO_4/1.2 mM KH_2PO_4/140 mM NaCl/10 mM KCl, pH 7.4/1.0 mM $CaCl_2$/0.5 mM $MgCl_2$). A 1:10 dilution of αId or NpS diluted in ice-cold binding medium (medium 199, 50 μg of bovine serum albumin per ml, 100 μg of gelatin per ml, with 20 mM Hepes, pH 7.4) was added to the cell monolayers that were then incubated for 2 hr at 4°C. DT diluted in binding medium was added, and the cells were incubated for 1 hr at 37°C. The monolayers were washed to remove unbound DT and further incubated in leucine-deficient medium. The cells were then incubated with [^3H]leucine for a final hour and subsequently washed, lysed, and counted for [^3H]leucine incorporation into trichloroacetic acid-precipitable material as described (17, 18). All assays were done in triplicate, and variation from the mean was 5–10%.

Titration of Guinea Pig Serum with Radioiodinated DT. DT was radioiodinated by the Iodogen method as described (9); the specific activity of the radioiodinated DT was typically $1–2 \times 10^7$ cpm/μg. Increased amounts of serum were added to radiolabeled DT in Tris-buffered saline (TBS; 10 mM Tris·HCl/150 mM NaCl, pH 7.5) with 0.1 mg of bovine serum albumin per ml and incubated 1 hr on ice. The mixture was then transferred to Pansorbin cells and incubated 15 min on ice. Pellets were washed with TBS containing bovine serum albumin, and the amount of immunoprecipitated radioactivity was determined.

DT-Binding Assay. Vero cells were plated and preincubated with guinea pig serum as described for the cytotoxicity assay. Cells were then washed and incubated with radiolabeled DT (25–250 ng/ml) in binding medium for 4 hr at 4°C. The cells were washed to remove unbound DT and solubilized in 0.1 M NaOH, and then cell-associated radioactivity was measured (19). Nonspecific binding was defined by the radioactive DT that remained associated with the cells when the radiolabeled DT incubation was done with 100-fold excess of unlabeled DT. All assays were done in duplicate, and variation from the mean was 5–10%.

Internalization Assay. Cell monolayers were incubated with a 1:10 dilution of αId or NpS for 2 hr at 4°C as described for cytotoxicity assay. Subsequently, ^{125}I-labeled DT (100 ng/ml) without or with a 200-fold excess of unlabeled DT (to determine nonspecific binding as defined above) was added, and the incubation was continued for 4 hr at 4°C. The monolayers were then washed to remove unbound DT, and the medium was replaced with Hanks' balanced salt solution (HBSS) containing 20 mM Hepes. Cell monolayers were further incubated at 37°C, and trichloroacetic acid-soluble and -insoluble material in the medium was determined at the time points indicated in the figure legend. Cell-associated radioactivity was determined by release of receptor-associated DT by Pronase and inositol hexaphosphate ($InsP_6$) treatment as described by Dorland *et al.* (20). Briefly, after the cells were washed, ice-cold HBSS containing 0.25 mg of Pronase per ml and 10 mg of $InsP_6$ per ml was added, and the cells were incubated 1 hr at 4°C. Heat-inactivated fetal bovine serum was then added to the cell monolayers to a final concentration of 50%. Cells were removed from the wells and separated by centrifugation, and the resulting supernatants and pellets were assayed for radioactivity. Pronase/$InsP_6$-releasable radioactivity represents surface-bound DT, whereas cell-associated radioactivity represents DT that is intracellular plus that amount of surface-bound DT not releasable by Pronase/$InsP_6$. Under these conditions, no greater than 85% of surface-bound material is removable (20, 21).

Immunoprecipitation of Surface-Labeled Vero Cell Lysates. Vero cells were surface labeled by the lactoperoxidase method and a Nonidet P-40 cell lysate prepared as described (9). The lysate was divided into two halves (1.5 ml and $\approx 1 \times 10^7$ cell equivalents each), and one-half was precleared by incubating with 100 μl of pooled normal guinea pig serum for 3 hr at 4°C followed by incubation with Pansorbin cells for 30 min at 4°C. Each half was further divided into two portions, and each portion was incubated with 50 μl of αId or pooled normal guinea pig serum overnight at 4°C with mixing. Samples were then added to Pansorbin cells and incubated for 30 min at 4°C. The resulting immune precipitates were washed and treated with electrophoresis treatment buffer and subjected to $NaDodSO_4$/PAGE and autoradiography as described (9).

RESULTS AND DISCUSSION

Effect of Anti-Idiotypic Sera on DT-Mediated Cytotoxicity. The αId generated was shown to be capable of inducing DT-neutralizing anti-anti-idiotypic antibodies (S.M.T. and A.B.M., unpublished work). This observation suggested that the αId contained an internal image of an epitope on DT that interacts with a cell-surface component essential for the DT-mediated intoxication process. Therefore, we tested the effect of αId in an *in vitro* cytotoxicity assay. Vero cells were chosen because they have been shown to be among the most DT-sensitive cells and to possess a high number of DT receptors (19). Fig. 1 shows that preincubation of Vero cells with αId followed by DT treatment resulted in a 33-fold protective effect [i.e., the concentration of toxin that inhibits protein synthesis by 50% (IC_{50}) was 1×10^{-5} mg/ml compared with an IC_{50} of 3×10^{-7} mg/ml when toxin alone was used]. This protective effect was *not* seen with NpS (Fig. 1), the hyperimmune anti-idiotypic serum incapable of inducing a DT-neutralizing anti-anti-idiotype.

Titration of αId with Radioiodinated DT. To ensure that the protective effect of αId was not from interaction of antibody with DT itself (22), the serum was tested for its ability to immunoprecipitate radioiodinated DT; αId was unable to precipitate DT to any detectable extent, even when the ratio αId:DT was 40-fold greater than the lowest ratio found to be protective in the cytotoxicity experiments (Fig. 1). Under the chosen conditions, guinea pig anti-diphtheria toxoid serum quantitatively precipitated the radioiodinated DT. This result suggests that αId protects cells from DT-mediated cytotoxicity not by binding to DT but by interacting with a Vero cell component involved in the intoxication process.

Effect of αId on DT Binding to Vero Cells. The initial step in DT-mediated intoxication of sensitive cells involves toxin binding to cell-surface receptors (1–4). Because αId was shown to protect cells from the effects of DT, apparently by interacting with a Vero cell component, the possibility

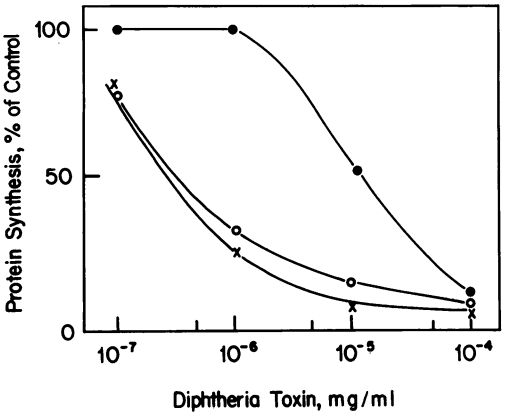

FIG. 1. Effect of αId treatment of Vero cells on the cytotoxic activity of DT. Vero cells were incubated for 2 hr at 4°C without hyperimmune guinea pig serum (×) or with αId (●) or NpS (○) after which time varying amounts of DT were added and the incubation was continued for 1 hr at 37°C. The cells were then washed to remove unbound DT, incubated for 1 hr with leucine-deficient medium, incubated for a final hr with [^{3}H]leucine, and assayed for trichloroacetic acid-precipitable radioactivity as described. The average amount of [^{3}H]leucine incorporation in the absence of DT was 35,610 cpm (100% of control activity).

existed that αId recognizes the DT receptor. Therefore, αId was next tested for its ability to prevent binding of radiolabeled DT to its receptor on Vero cells. αId or NpS was preincubated with the cells as in the cytotoxicity protection experiments, the cells were washed with PBS/$CaCl_2$/$MgCl_2$, and increasing concentrations of radiolabeled DT were allowed to bind to the cells. After unbound DT was washed away, cell-associated radioactivity was assessed. At all DT concentrations tested, the specific as well as the nonspecific binding of DT to Vero cells was similar when the cells were preincubated with αId, NpS, or guinea pig anti-tetanus toxoid, an irrelevant control serum (data not shown). Under these conditions, therefore, αId is unable to prevent the binding of DT to its receptor, suggesting that the internal image represented by αId does not correspond to the receptor-binding site on DT.

Effect of αId on the Internalization of DT. The fact that DT-mediated cytotoxicity was decreased in the presence of αId, although this same serum could not inhibit the binding of radiolabeled DT to intact cells suggested that αId may affect a step subsequent to toxin binding. Therefore, the kinetics of toxin internalization in the presence of αId were examined. Preliminary experiments indicated that coincubation of αId with radiolabeled DT, under conditions known to allow toxin internalization and degradation (20, 21) caused most DT to remain cell associated. To determine whether the cell-associated DT, in the presence of αId, remains surface-bound or resides in internal compartments, the fate of radiolabeled DT was followed and was compared with the fate of the toxin in the presence of NpS as described. Fig. 2 shows that after 3 hr at 37°C in the presence of NpS, most radiolabeled DT bound at t_0 was rapidly internalized, degraded, and returned to the medium as trichloroacetic acid-soluble fragments (Fig. 2*A*). In contrast, when radiolabeled DT was bound in the presence of αId, the majority of DT remained cell-surface bound, and the percentage of DT degraded after internalization was greatly reduced (Fig. 2*B*). This result suggests that αId protects Vero cells by interacting with a cell-surface component, affecting a step in the intoxication process subsequent to DT binding.

Immunoprecipitation of Surface-Labeled Vero Cell Lysates with αId. In an attempt to identify the Vero cell-surface component recognized by αId, surface-labeled Vero cells were lysed in nonionic detergent, half of the lysate was

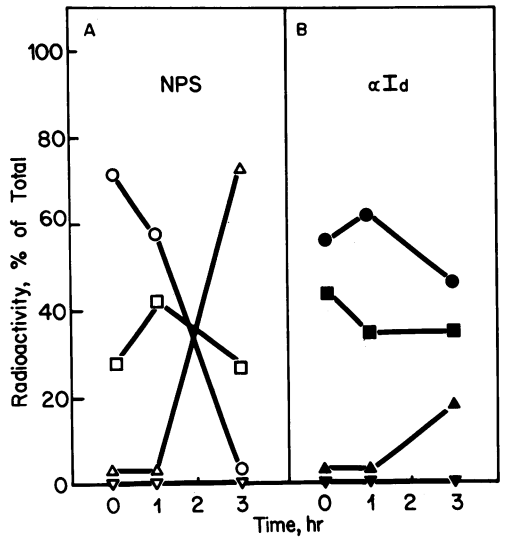

FIG. 2. Effect of αId on the internalization of DT. Vero cells were incubated for 2 hr at 4°C with NpS (NPS) (*A*) or αId (*B*). Subsequently, ^{125}I-labeled DT (100 ng/ml) in the presence or absence of a 200-fold excess of unlabeled DT was added, and the incubation was continued for 4 hr at 4°C. Cell monolayers were washed and further incubated at 37°C. At the indicated times, radioactivity was separated into four fractions as described, corrected for nonspecific binding, and graphed as the percent of the total at each time. Total radioactivity represents the following fractions: surface-associated DT released by Pronase/InsP_6 treatment (○, ●); cell-associated DT—that is, intracellular DT plus that amount of surface-bound DT not released by Pronase/InsP_6 (□, ■); acid-soluble, degraded DT in the medium (△, ▲); and acid-precipitable DT in the medium (▽, ▼).

precleared by incubation with pooled normal (preimmune) guinea pig serum followed by protein A-bearing *Staphylococcus aureus*, and the precleared and nonprecleared lysates were incubated with αId or pooled normal guinea pig serum, as described. Immune complexes were precipitated with protein A-bearing *S. aureus* and were analyzed by NaDodSO$_4$/PAGE and autoradiography. When the nonprecleared immune precipitates are examined, the αId precipitate reveals an enrichment in low-molecular weight proteins compared with the normal guinea pig serum control (Fig. 3*A*, lanes 2 and 3). Immune precipitates from lysates precleared before immunoprecipitation show a reduced number of nonspecifically precipitated proteins and more distinct immune precipitate profiles. The αId precipitate still reveals an enhanced immunoprecipitation of low-molecular weight material, particularly a protein of $M_r \approx 15{,}000$, when compared to the control (Fig. 3*B*, lanes 2 and 3). The mass of the protein of $M_r \approx 15{,}000$ immunoprecipitated by αId is unchanged when the samples are analyzed under nonreducing conditions (data not shown). Because αId prevents the internalization of DT interacting with a Vero cell component, this radiolabeled Vero cell-surface protein immunoprecipitated by αId could represent a cell component essential to the internalization process. Furthermore, αId must constitute an internal image of a DT site important in the internalization process of the toxin.

Our results are consistent with a model in which the αId protects Vero cells by binding to a cell-surface protein involved in the intoxication process at a step subsequent to the initial binding of the toxin. Whether the cell-surface protein of $M_r \approx 15{,}000$ detected by immunoprecipitation with αId is the toxin receptor itself or another cell-surface protein remains to be established. Nevertheless, the second cell-surface protein (or second site on the receptor) recognized by αId must possess a site complementary to an epitope on the toxin; this toxin epitope is not the receptor-binding site but

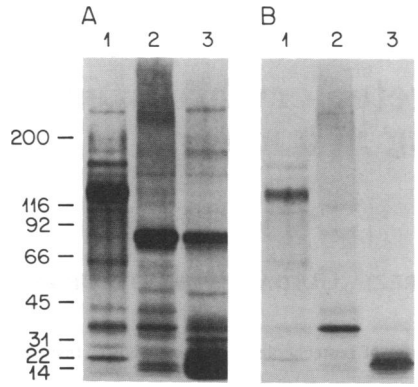

FIG. 3. Immunoprecipitation of surface-labeled Vero cell lysates with αId. Vero cells were surface-labeled with $Na^{125}I$, lysed in Nonidet P-40, and imunoprecipitated with guinea pig sera as described. (*A*) Immune precipitates were formed by incubating test serum with cell lysates immediately after detergent extraction of the cells. (*B*) Cell lysates were first precleared with pooled normal guinea pig serum before incubation with the test serum. Samples were reduced with 5% 2-mercaptoethanol before $NaDodSO_4$/PAGE on a 4–15% gradient gel. The sample order in *A* and *B* is identical. Lanes: 1, crude detergent extract of cells; 2, immune precipitate from cell lysate incubated with pooled normal guinea pig serum; 3, immune precipitate from cell lysate incubated with αId. Within each panel, an equivalent amount of radioactivity from each sample (lanes 1, 2, and 3) was analyzed.

rather a site involved in internalization, possibly located in the middle of the B fragment (6).

The existence of a second cell-surface site involved in DT intoxication has previously been suggested. (*i*) Our laboratory (23), based on biochemical experiments with solubilized DT-binding cell-surface proteins, suggested the existence of such a site on the receptor (X′ site) that interacts with a site on the toxin (X site) distinct from the receptor-binding site and postulated that the X′ site is involved in a step subsequent to the initial toxin binding. (*ii*) Didsbury *et al.* (24) suggested the existence of a second function for the receptor, or a second site, based on a distinct class of DT-resistant Chinese hamster ovary cells (Dip^r) that bind toxin normally but are 10,000-fold more resistant to DT than are the wild-type cells. These investigators proposed that this class of mutants has a block at a step in the intoxication process that is between toxin binding and its pH-dependent entry into the cytosol from an intracellular vesicle (24).

The observation that the αId retained the toxin on the cell surface—i.e., accessible to cleavage by Pronase—strongly suggests that the cell-surface component affected by αId is involved in an early step (subsequent to toxin binding) in the intoxication process, possibly the movement of the receptor into coated pits or its subsequent internalization from coated pits. Use of anti-idiotypic antibodies should allow the further characterization of cellular components involved in receptor-mediated endocytosis of DT.

We thank J. Donald Capra, Robert S. Munford, R. Jerrold Fulton, and Joseph G. Naglich for critical review of the manuscript. The secretarial help of Cindy Baselski and the editorial assistance of Eleanor R. Eidels are appreciated. This research was supported by U.S. Public Health Service Grants AI-16805, AI-24760, and EY-03094 from the National Institutes of Health and by a grant from the Mobay Corporation, Animal Health Division, Shawnee Mission, KS. J.M.R. was supported, in part, by Cancer Immunology Training Grant CA-09082 and by a grant from the Texas Department of the Ladies Auxiliary, Veterans of Foreign Wars.

1. Collier, R. J. (1975) *Bacteriol. Rev.* **39**, 54–85.
2. Pappenheimer, A. M., Jr. (1977) *Annu. Rev. Biochem.* **46**, 69–94.
3. Eidels, L., Proia, R. L. & Hart, D. A. (1983) *Microbiol. Rev.* **47**, 596–620.
4. Middlebrook, J. L. & Dorland, R. B. (1984) *Microbiol. Rev.* **48**, 199–221.
5. Falmagne, P., Capiau, C., Lambotte, P., Zanen, J., Cabiaux, V. & Ruysschaert, J.-M. (1985) *Biochim. Biophys. Acta* **827**, 45–50.
6. Hayakawa, S., Uchida, T., Mekada, E., Moynihan, M. R. & Okada, Y. (1983) *J. Biol. Chem.* **258**, 4311–4317.
7. Keen, J. H., Maxfield, F. R., Hardegree, M. C. & Habig, W. H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2912–2916.
8. Morris, R. E., Gerstein, A. S., Bonventre, P. F. & Saelinger, C. B. (1985) *Infect. Immun.* **50**, 721–727.
9. Cieplak, W., Gaudin, H. M. & Eidels, L. (1987) *J. Biol. Chem.* **262**, 13246–13253.
10. Mekada, E., Okada, Y. & Uchida, T. (1988) *J. Cell Biol.* **107**, 511–519.
11. Sege, K. & Peterson, P. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2443–2447.
12. Schreiber, A. B., Couraud, P. O., Andre, C., Vray, B. & Strosberg, A. D. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7385–7389.
13. Lambris, J. D. & Ross, G. D. (1982) *J. Exp. Med.* **155**, 1400–1411.
14. Islam, M. N., Pepper, B. M., Briones-Urbina, R. & Farid, N. R. (1983) *Eur. J. Immunol.* **13**, 57–63.
15. Pain, D., Kanwar, Y. S. & Blobel, G. (1988) *Nature (London)* **331**, 232–237.
16. Pappenheimer, A. M., Jr., Uchida, T. & Harper, A. A. (1972) *Immunochemistry* **9**, 891–906.
17. Proia, R. L., Eidels, L. & Hart, D. A. (1981) *J. Biol. Chem.* **256**, 4991–4997.
18. Eidels, L. & Hart, D. A. (1982) *Infect. Immun.* **37**, 1054–1058.
19. Middlebrook, J. L., Dorland, R. B. & Leppla, S. H. (1978) *J. Biol. Chem.* **253**, 7325–7330.
20. Dorland, R. B., Middlebrook, J. L. & Leppla, S. H. (1979) *J. Biol. Chem.* **254**, 11337–11342.
21. Marnell, M. H., Shia, S.-P., Stookey, M. & Draper, R. K. (1984) *Infect. Immun.* **44**, 145–150.
22. Schechter, Y., Maron, R., Elias, D. & Cohen, I. R. (1982) *Science* **216**, 542–545.
23. Eidels, L., Ross, L. L. & Hart, D. A. (1982) *Biochem. Biophys. Res. Commun.* **109**, 493–499.
24. Didsbury, J. R., Moehring, J. M. & Moehring, T. J. (1983) *Mol. Cell. Biol.* **3**, 1283–1294.