Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 6894–6898, November 1982
Biochemistry

Preparation, characterization, and properties of monoclonal antibodies against the *lac* carrier protein from *Escherichia coli*

(radioimmunoassay/immunoblotting/active transport/membrane vesicles/proteoliposomes)

NANCY CARRASCO, STANLEY M. TAHARA, LEKHA PATEL, TZIPORA GOLDKORN, AND H. RONALD KABACK*

Laboratory of Membrane Biochemistry, Roche Institute of Molecular Biology, Nutley, New Jersey 07110

Communicated by Herbert Weissbach, August 20, 1982

ABSTRACT **Monoclonal antibodies directed against the *lac* carrier protein purified from the membrane of *Escherichia coli* were prepared by somatic cell fusion of mouse myeloma cells with splenocytes from an immunized mouse. Several clones produce antibodies that react with the purified protein as demonstrated by solid-phase radioimmunoassay and by immunoblotting experiments; culture supernatants from the clones inhibit active transport of lactose in isolated membrane vesicles. Five stable clones were selected for expansion, formal cloning, and production of ascites fluid, and the antibodies secreted *in vivo* by each clone also were found to inhibit lactose transport. Antibody from hybridoma 4B1, an IgG2a immunoglobulin, inhibits active transport of lactose in proteoliposomes reconstituted with purified *lac* carrier and in right-side-out membrane vesicles. In contrast, the antibody has no effect on the generation of the proton electrochemical gradient by membrane vesicles nor does it alter the ability of vesicles containing the *lac* carrier to bind *p*-nitrophenyl-α-D-galactopyranoside. In order to achieve 50% inhibition of transport activity, a 2- to 3-fold molar excess of antibody to *lac* carrier is required, regardless of the amount of *lac* carrier in the membrane. Thus, the concentration of antibody required for a given degree of inhibition is proportional to the amount of *lac* carrier in the membrane. Finally, antibody-induced inhibition occurs within seconds, an observation suggesting that the epitope is accessible on the surface of the membrane.**

Transport of β-galactosides in *Escherichia coli* is catalyzed by the product of the *lac y* gene (1), the *lac* carrier protein, which translocates β-galactosides with protons in a symport reaction (2). Accordingly, in the presence of a proton electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$), hydrogen ion moves down the electrochemical gradient and drives the uphill translocation of sugar (see ref. 3 for a review). The *lac* carrier was identified as a membrane-bound protein chemically in 1965 (4) and functionally in 1970 (5). Eight years later in rapid succession, the *lac y* gene was cloned in a recombinant plasmid, its product was amplified (6) and synthesized *in vitro* (7), and the sequence of the protein was deduced from the DNA sequence (8). Shortly thereafter, it was demonstrated (9) that lactose transport activity can be solubilized and reconstituted into proteoliposomes, and a highly specific photoaffinity label for the *lac* carrier was developed (10). Most recently, use of these techniques has led to the purification of a single protein, its identification as the product of the *lac y* gene, and the demonstration that it is the only polypeptide in the cytoplasmic membrane required for lactose/proton symport (11, 12).

The *lac* carrier protein is a 46.5 kilodalton (kDal) polypeptide chain of 417 amino acid residues of known sequence (8, 11). A preliminary secondary structure model for the molecule has been formulated (unpublished data) based on the hydropathic nature of the protein along its sequence (13) and on the observation that the protein is ≈85% α-helical as determined by circular dichroism. In the model, the protein consists of at least 12 α-helical segments that traverse the lipid bilayer in a manner similar to that suggested for bacteriorhodopsin (14). In addition, proteolysis experiments with right-side-out and inverted vesicles, in which the *lac* carrier was specifically photolabeled with *p*-nitrophenyl α-D-galactopyranoside (NPG), demonstrate that the protein spans the bilayer and that the binding site is contained in a transmembrane segment (15). Finally, detailed kinetic studies (16, 17) and radiation inactivation analysis (18), coupled with the observation that certain *lac y* gene mutations are dominant (19), are consistent with the notion that $\Delta\tilde{\mu}_{H^+}$ may induce a major alteration in subunit interaction (e.g., dimerization).

In order to test these proposals, a variety of experimental approaches are required, one of which is the use of highly specific probes for topology and structure–function relationships. A class of molecules that has this potential is monoclonal antibodies (20), and in this communication, we report the preparation and preliminary characterization of such antibodies directed against purified *lac* carrier protein.

MATERIALS AND METHODS

Purification and Reconstitution of *lac* Carrier Protein. The *lac* carrier protein was purified and reconstituted into proteoliposomes by a modification (12) of the original procedure (11). The sample used for immunization was obtained directly from the DEAE-Sepharose column in the last step of purification (11), dialyzed overnight against distilled water, lyophilized, and resuspended in phosphate-buffered saline (P_i/NaCl; 10 mM sodium phosphate, pH 7.2/150 mM NaCl) to a final concentration of 250 μg of protein and 415 μg of *E. coli* phospholipid per ml.

Construction of Hybridoma Cells and Antibody Production. BALB/c female mice (Charles River Breeding Laboratories) were immunized with 25 μg of purified *lac* carrier protein. An aliquot of the immunogen (0.1 ml) was emulsified with 0.1 ml of complete Freund's adjuvant and injected subcutaneously. On day 24, serum samples obtained from the mice were tested for the presence of antibodies by solid-phase radioimmunoassay (SP-RIA). One mouse whose serum was positive was given booster injections intraperitoneally on days 29 and 43 with 50 μg of antigen in 0.2 ml of P_i/NaCl. Three days after the last

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.

Abbreviations: $\Delta\tilde{\mu}_{H^+}$, proton electrochemical gradient; NPG, *p*-nitrophenyl α-D-galactopyranoside; P_i/NaCl, phosphate-buffered saline; SP-RIA, solid-phase radioimmunoassay; kDal, kilodaltons.

* To whom reprint requests should be addressed.

injection, the spleen was removed aseptically, and spleen cells were fused with P3X63Ag8.653 myeloma cells by using 50% polyethylene glycol 4000 (Merck) (21). The myeloma cells were maintained in RPMI 1640 growth medium supplemented with 15% heat-inactivated fetal calf serum (GIBCO), 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 100 units of penicillin and 100 μg of streptomycin per ml.

Hybridoma cells were selected with RPMI 1640 medium containing hypoxanthine, aminopterin, and thymidine (22). Subcloning of positive wells was performed in two stages as described (23), with the exception that mouse macrophages were used as a feeder layer in place of mouse thymocytes. Selected hybridoma formal clones were expanded for ascites tumor production and frozen as reported (22).

Production of Ascites Fluid. For large-scale antibody production, ≈5–7 × 10^6 hybridoma cells in 1.0 ml of P_i/NaCl were injected intraperitoneally in BALB/c mice primed at least 14 days earlier with 0.5 ml of pristane (Aldrich). Ascites fluid was collected after 10–15 days, clarified by centrifugation, and stored at −20°C.

Purification of Antibodies. Antibody contained in ascites fluids was purified by affinity chromatography on protein A-Sepharose (24). An aliquot containing ≈100 mg of protein was applied to the column and washed with 0.1 M sodium phosphate (pH 8.1) until no protein was detected in the eluant. The bound immunoglobulins were then eluted stepwise with sodium citrate buffer at pH 6.0, 5.5, 4.5, and 3.5, respectively. Proteins eluted from the column were concentrated by evaporation under vacuum and dialyzed overnight against 1,000 vol of 0.1 M potassium phosphate (pH 7.5) with two changes. Aliquots of purified antibodies were frozen and stored in liquid nitrogen.

SP-RIA. All manipulations were performed at room temperature. Purified *lac* carrier (0.1–0.2 μg) was applied in a 5-μl aliquot to each well of a Millititer plate (Millipore) and allowed to adsorb for 30 min. Nonspecific protein-binding sites were then blocked by adding 400 μl of 5% bovine serum albumin in 10 mM Tris·HCl, pH 7.4/0.9% NaCl, referred to as Tris/saline. The albumin solution was shaken out of the wells, and 50 μl of tissue culture supernatant, ascites fluid, or purified antibody was added, followed by a 2-hr incubation. Antibody-containing solutions were removed by aspiration, and the wells were washed several times with Tris/saline, followed by several washes with Tris/saline containing 0.05% Nonidet P-40. ^{125}I-Labeled protein A (10^5 cpm; prepared as described in ref. 23) was added to each well in 50 μl of 1% bovine serum albumin in P_i/NaCl, and the plates were incubated for 60 min. Unbound ^{125}I-labeled protein A was removed, and the wells were washed as before. Bound radioactivity was detected by autoradiography at −70°C with Kodak XAR-5 film and a Cronex intensifier screen (DuPont).

Immunoblotting. Purified *lac* carrier or membrane vesicles were electrophoresed on $NaDodSO_4$/polyacrylamide gels (11), and the protein bands were transferred to nitrocellulose (BA85; Schleicher & Schuell) either electrophoretically (purified *lac* carrier) or by diffusion (membrane vesicles). Electroblotting was carried out for 7 hr at constant voltage (12 V; *ca.* 200 mA) as described (25), except that the transfer buffer was supplemented with 0.5% aminoxid WS-35 (Th. Goldschmidt AG, Essen, Federal Republic of Germany). Diffusion blotting (26) was carried out for 72 hr in the same buffer system, but 0.6% octyl-β-D-glucopyranoside was used in place of aminoxid. After transfer, the nitrocellulose sheets were sliced into vertical strips and used directly for immunoassay (27).

Growth of Bacteria and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) and *E. coli* T206, which carries the *lac y* gene in a recombinant DNA plasmid, were grown as described (refs. 28 and 16, respectively), and right-side-out membrane vesicles were prepared by osmotic lysis (29, 30).

Transport Assays. Transport of [1-^{14}C]lactose was measured under oxygen with reduced phenazine methosulfate as electron donor (31).

Protein. Protein was determined as described (32) with crystalline bovine serum albumin as standard.

RESULTS

Preparation and Characterization of Monoclonal Antibodies Against the *lac* Carrier. Of three BALB/c mice immunized with purified *lac* carrier protein, a single animal exhibited a positive serum response. After further booster injections, spleen cells were obtained from this mouse, fused with P3X63Ag8.653 myeloma cells and plated into five 96-well culture dishes (446 wells total). After 15 days in culture, 142 wells (32%) showed sufficient cell growth to assay for antibody. Because the *lac* carrier protein does not adsorb to polystyrene or polyvinyl chloride, conventional SP-RIA was not feasible; however, the protein adsorbs to nitrocellulose, and Millititer plates were used for the assay. From the film densities, ≈59 wells (42%) were deemed positive, and cells from these wells were expanded into 24-well plates. After sufficient growth had occurred, supernatant fluids were tested for antibody specificity by immunoblotting against purified *lac* carrier and against T206 membrane vesicles. All of the hybridomas chosen for expansion reacted with the major polypeptide in the purified *lac* carrier preparation that migrated at M_r 33,000 (Fig. 1).† In addition, a protein of higher apparent M_r (65,000) also reacted with the supernatants. Because the same bands were observed with purified *lac* carrier photolabeled with [^{3}H]NPG (unpublished observations), it is likely that the material at M_r 65,000 represents an aggregate of the *lac* carrier. In any case, essentially identical results were obtained with T206 membrane vesicles (Fig. 1), providing even stronger evidence for the high degree of specificity of the antibodies for the *lac* carrier. Control assays with culture supernatants from the parent myeloma line were negative, indicating that the reactions observed are dependent on hybridoma antibodies.

Hybridomas 3G12, 4A10R, 4B1, 4B11, and 5F7 were subcloned by limiting dilution in two stages (23). Cloned cells were expanded in culture and injected into pristane-primed mice for ascites fluid production, and the antibodies therein were purified by chromatography on protein A-Sepharose (24). Fractions from the affinity column were tested by SP-RIA and the lactose transport assay (see below), and those with activity were analyzed for IgG subclass by Ouchterlony double-diffusion analysis with known rabbit antisera against different mouse IgG subclasses. Antibodies secreted by hybridomas 4B1, 4B11, and 5F7 were found to be of the IgG2a subclass, whereas clones 4A10R and 3G12 produced IgG3 immunoglobulins.

Effect of Monoclonal Antibodies on Lactose Transport. In order to correlate immunochemical properties with function, supernatants from hybridomas at the 24-well stage were screened initially for effects on lactose transport in isolated membrane vesicles. Both the initial rate of transport and the steady-state level of accumulation were inhibited 20–90%, depending on the particular supernatant, and antibody 4B1 showed the most pronounced effect (data not shown). Subsequently, antibodies were purified from the five hybridoma

† Although the M_r of the *lac* carrier protein is 46,504, as determined from the DNA sequence of the *lac y* gene (8) and from the amino acid composition of the purified carrier (11), for unknown reasons, the protein migrates with an apparent M_r of ≈33,000 during electrophoresis in 12% $NaDodSO_4$/polyacrylamide gels (see ref. 11).

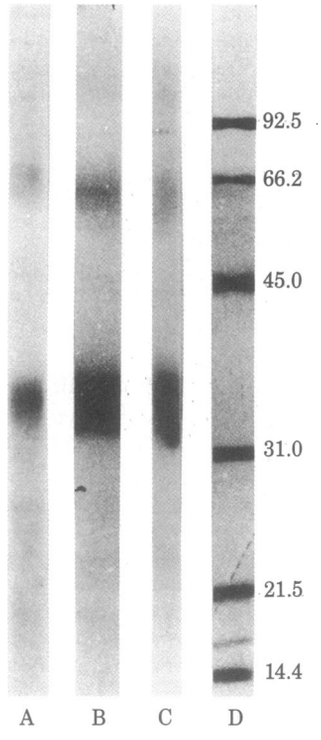

FIG. 1. Specificity of 4B1 hybridoma supernatant for the *lac* carrier protein. Purified *lac* carrier protein and right-side-out T206 membrane vesicles extracted with 5 M urea were electrophoresed on 12% $NaDodSO_4$/polyacrylamide gels as described (11). Protein bands were then transferred to nitrocellulose by electroblotting (*lac* carrier protein) or diffusion blotting (T206 vesicles). Individual strips were incubated with 4B1 hybridoma supernatant for 2 hr at room temperature, followed by washing and incubation with ^{125}I-labeled protein A (*ca.* 2×10^5 cpm per strip). Autoradiography was carried out at −70°C for ≈5 hr. Lanes: A, purified *lac* carrier protein after $NaDodSO_4$/polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue; B and C, autoradiograms of immunoblotted *lac* carrier protein and T206 membrane vesicles, respectively; D, M_r standards after $NaDodSO_4$/polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue (phosphorylase, 92.5 kDal; BSA, 66.2 kDal; ovalbumin, 45.0 kDal; carbonic anhydrase, 31.0 kDal; soybean trypsin inhibitor, 21.5 kDal; and lysozyme, 14.4 kDal).

clones and retested for inhibitory activity, and 4B1 antibody was observed again to be most potent. Antibody from this hybridoma clone was selected for more detailed study.

Proteoliposomes reconstituted with purified *lac* carrier catalyze lactose transport when a membrane potential, $\Delta\Psi$ (interior negative), is imposed by means of a potassium diffusion gradient in the presence of valinomycin (refs. 11 and 12; Fig. 2). When antibody 4B1 was added to the proteoliposomes, the initial rate of transport was inhibited by ≈80%, whereas addition of IgG2a monoclonal antibody against D-lactate dehydrogenase‡ or an IgG2a produced by UPC 10 mouse myeloma cells had no significant effect.

E. coli T206 harbors a multiple-copy hybrid plasmid encoding the *lac y* gene (28), and membranes from this strain show 5–6 times the amount of *lac* carrier protein as do membranes from strains such as ML 308-225 that contain only one gene copy (28, 34). When initial rates of respiration-driven lactose transport in membrane vesicles from ML 308-225 and T206 were measured as a function of 4B1 antibody concentration, different

‡ Monoclonal antibodies against D-lactate dehydrogenase purified from *E. coli* membranes (33) were prepared and characterized by methods similar to those described here (unpublished data).

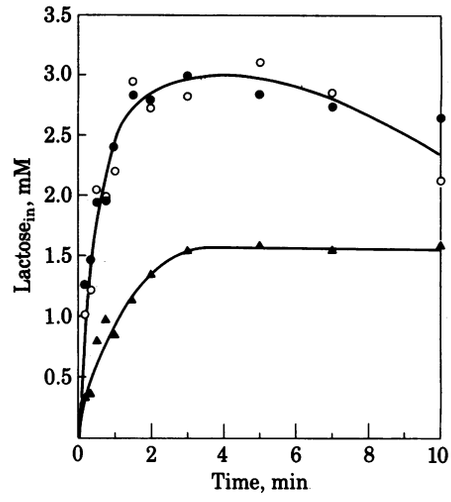

FIG. 2. Effect of antibody 4B1 on membrane potential ($\Delta\Psi$)-driven lactose transport in proteoliposomes reconstituted with purified *lac* carrier protein. The *lac* carrier protein was purified, reconstituted into proteoliposomes, and assayed as described by Newman *et al.* (11). Proteoliposomes containing ≈2.5 μg of *lac* carrier protein were incubated for 30 min at room temperature with 25 μg of antibody 4B1 (≈3-fold molar excess of antibody over *lac* carrier protein) in a total volume of 78 μl. Control samples were treated identically, except that they contained 25 μg of IgG2a monoclonal antibody against D-lactate dehydrogenase (33) or 25 μg of an IgG2a produced by UPC 10 mouse myeloma cells. The samples were then centrifuged in a Beckman Airfuge at about 160,000 × *g* for 70 min, and the supernatants were discarded. The pellets were resuspended in 28 μl of 50 mM potassium phosphate, pH 7.5/1 mM dithiothreitol, and 20 μM valinomycin was added (final concentration). Lactose transport was measured as described (11) by diluting 1 μl of proteoliposomes into 200 μl of 50 mM sodium phosphate (pH 7.5) containing 0.3 mM [1-^{14}C]lactose (19 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels). ▲, Proteoliposomes treated with antibody 4B1; ○, untreated proteoliposomes; ●, proteoliposomes treated with either monoclonal antibody against D-lactate dehydrogenase or IgG2a from UPC 10 cells. Internal lactose concentration is plotted as a function of time. The internal volume of the proteoliposomes was determined from the trapped volume of [1-^{14}C]lactose after passive equilibration and filtration at 0°C (unpublished data).

concentrations of antibody were required to achieve the same degree of inhibition, and a maximum of 80% inhibition was observed in both preparations (Fig. 3). With ML 308-225 vesicles, 50% inhibition was observed at about 0.7 μg of antibody per assay, whereas 3.5 μg per assay was required for 50% inhibition with T206 vesicles. Clearly, this difference corresponds reasonably well with the difference in the concentration of *lac* carrier in the two membranes. Moreover, by assuming that the *lac* carrier represents 0.2% and 1.2% of the membrane protein in ML 308-225 and T206, respectively, and that the M_rs of the *lac* carrier protein and IgG2a are 46.5 and 150 kDal, respectively, it can be calculated that the molar ratio of antibody to *lac* carrier is 2–3 at 50% inhibition in both membrane preparations. In other words, the amount of antibody required for inhibition appears to be directly related to the quantity of *lac* carrier in the membrane.

Although data will not be presented, incubation of vesicles with 4B1 antibody at concentrations that induce maximum inhibition of transport had no effect whatsoever on the ability of the vesicles to generate $\Delta\bar{\mu}_{H^+}$, as judged by flow dialysis experiments with [^{3}H]tetraphenylphosphonium ion at pH 7.5 (35, 36). Furthermore, despite potent inhibition of lactose transport, the antibody had no effect on [^{3}H]NPG binding under nonenergized conditions when assayed either by flow dialysis (37) or photolabeling (10).

The time course of inhibition induced by 4B1 antibody was

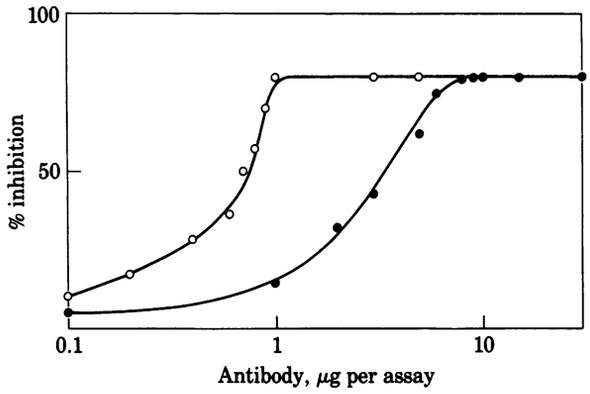

FIG. 3. Inhibitory effect of antibody 4B1 on initial rates of respiration-driven lactose transport in membrane vesicles from ML 308-225 (○) and T206 (●). Aliquots (10 μl) of right-side-out membrane vesicles containing 40 μg of membrane protein were diluted to a final volume of 50 μl containing 50 mM potassium phosphate (pH 7.5), 10 mM magnesium sulfate, and given amounts of antibody 4B1. The samples were incubated at 25°C for 10 min, and transport of 0.4 mM [1-^{14}C]lactose (59.9 mCi/mmol) was measured for 5 sec in the presence of ascorbate and phenazine methosulfate as described (31). Results are presented as percentage inhibition relative to control samples incubated in the absence of antibody.

extremely rapid (Fig. 4). In the experiment shown, ML 308-225 vesicles were treated with a 3- to 4-fold molar excess of antibody over *lac* carrier; at given times, the samples were diluted 1:20 to diminish further antigen–antibody reaction. Subsequently, the vesicles were recovered by centrifugation and assayed for

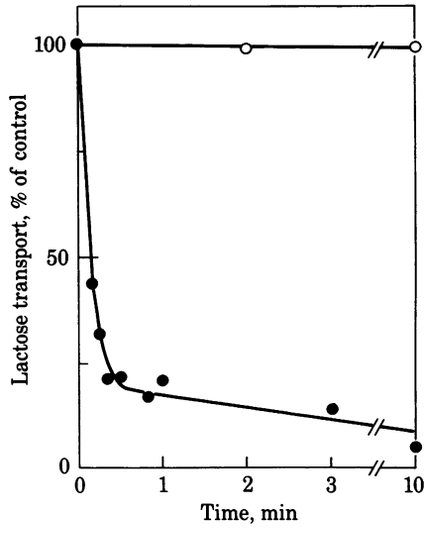

FIG. 4. Time course of antibody 4B1 inhibition of lactose transport. Aliquots of ML 308-225 membrane vesicles containing 0.6 mg of membrane protein were incubated with 0.01 mg of antibody 4B1 (a 3- to 4-fold molar excess of antibody over *lac* carrier protein) in a total volume of 0.5 ml under the conditions described in Fig. 3 (●). Alternatively, 0.01 mg of monoclonal antibody against D-lactate dehydrogenase (33) or antibody 4B1 that had been incubated in boiling water for 2 min was used in place of antibody 4B1 (○). At given times, the samples were diluted with 10 ml of 100 mM potassium phosphate (pH 7.5) to prevent further antigen–antibody reaction and immediately centrifuged at 40,000 × *g* for 30 min. After discarding the supernatants, the pellets were resuspended in 0.15 ml of 100 mM potassium phosphate (pH 7.5) and assayed for lactose transport for 5 sec in the presence of reduced phenazine methosulfate as described in Fig. 3. Results are presented as a percentage of control samples that were incubated with buffer only prior to centrifugation, resuspension, and assay.

lactose transport. Within 10 sec, >50% inhibition was observed, and by 30–60 sec, inhibition was essentially complete. In contrast, control vesicles treated under identical conditions with either IgG2a monoclonal antibody against D-lactate dehydrogenase‡ or boiled 4B1 antibody exhibited no loss of activity.

DISCUSSION

The results describe the preparation and preliminary characterization of monoclonal antibodies directed against the *lac* carrier protein purified from the membrane of *E. coli*. As judged by many criteria, the antibodies are highly specific for the *lac* carrier. (*i*) Antiserum from a mouse immunized with the carrier reacts with the purified protein immobilized on nitrocellulose. (*ii*) After the initial plating, hybridoma cells secrete antibodies that react exclusively with the *lac* carrier, as evidenced by immunoblotting studies with the purified carrier and with T206 membrane vesicles. (*iii*) Monoclonal antibodies produced *in vitro* and *in vivo* block lactose transport in right-side-out membrane vesicles, and antibody 4B1, an IgG2a secreted by a cloned hybridoma line, does not alter the generation of $\Delta\tilde{\mu}_{H^+}$. (*iv*) Antibody 4B1 inhibits lactose transport in proteoliposomes reconstituted with purified *lac* carrier protein. (*v*) The quantity of 4B1 antibody required for inhibition of lactose transport in membrane vesicles is proportional to the amount of *lac* carrier in the membrane.

Evidence is also available (not shown) that the antibody secreted by hybridoma 4B1 is a single molecular species of IgG. Antibody secreted by 4B1 cells before and after successive cloning exhibits identical properties both immunochemically and with regard to inhibition of transport. In addition, culture supernatants from hybridoma 4B1 show a single immunoprecipitin line on Ouchterlony double diffusion against known anti-IgG2a, and no reaction is observed with antibodies against other IgG subclasses. Finally, one- and two-dimensional electrophoretic analyses of supernatants from 4B1 hybridoma cells grown in the presence of [^{35}S]methionine reveal a single species of IgG heavy chain.

Although the binding constant of 4B1 antibody for the *lac* carrier has yet to be determined with precision, the antibody probably has a relatively high affinity. Inhibition of transport occurs within seconds after addition of antibody (Fig. 4), maximum inhibition is observed at a 3–4 molar excess of antibody to *lac* carrier (Fig. 3), and inhibition is not reversed by dilution and washing of vesicles after treatment with antibody. Furthermore, the rapid time course of inhibition suggests that the epitope in the *lac* carrier protein is readily accessible from the aqueous phase. Notably, however, studies with NPG, a high-affinity ligand for the *lac* carrier (10, 37), indicate that antibody 4B1 does not block the ability of the carrier to bind substrate. Given these observations and the suggestion that $\Delta\tilde{\mu}_{H^+}$ may induce a major conformational alteration in the *lac* carrier (e.g., dimerization) (16–18), it is tempting to speculate that antibody 4B1 might inhibit active lactose transport by preventing this conformational transition. In any event, further characterization of these antibodies combined with detailed structure–function studies in isolated membrane vesicles and reconstituted proteoliposomes may provide important insight into the mechanism of lactose/proton symport.

We are indebted to Drs. M.-L. Garcia, D. L. Foster, and P. V. Viitanen for purifying and reconstituting the *lac* carrier and to Dr. M.-L. Garcia for performing the experiment presented in Fig. 2. We also thank Dr. Aaron Shatkin for providing encouragement and tissue culture facilities during the inception of these experiments and Ms. Helena Champion and the Millipore Corporation for graciously providing Mil-

lititer plates. N.C. is a Research Fellow of the Fogarty International Center, National Institutes of Health, Bethesda, MD.

1. Cohen, G. N. & Monod, J. (1957) *Bacteriol. Rev.* **21,** 169–194.
2. Mitchell, P. (1963) *Biochem. Soc. Symp.* **22,** 142–168.
3. Kaback, H. R. (1981) in *Chemiosmotic Proton Circuits in Biological Membranes,* eds. Skulachev, V. P. & Hinkle, P. C. (Addison-Wesley, Reading, MA), pp. 525–536.
4. Fox, C. F. & Kennedy, E. P. (1965) *Proc. Natl. Acad. Sci. USA* **54,** 891–899.
5. Barnes, E. M. & Kaback, H. R. (1970) *Proc. Natl. Acad. Sci. USA* **66,** 1190–1198.
6. Teather, R. M., Müller-Hill, B., Abrutsch, U., Aichele, G. & Overath, P. (1978) *Mol. Gen. Genet.* **159,** 239–248.
7. Ehring, R., Beyreuther, K., Wright, J. K. & Overath, P. (1980) *Nature (London)* **283,** 537–540.
8. Büchel, D. E., Gronenborn, B. & Müller-Hill, B. (1980) *Nature (London)* **283,** 541–545.
9. Newman, M. J. & Wilson, T. H. (1980) *J. Biol. Chem.* **255,** 10583–10586.
10. Kaczorowski, G. J., LeBlanc, G. & Kaback, H. R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 6319–6323.
11. Newman, M. J., Foster, D. L., Wilson, T. H. & Kaback, H. R. (1981) *J. Biol. Chem.* **256,** 11804–11808.
12. Foster, D. L., Garcia, M.-L., Newman, M. J., Patel, L. & Kaback, H. R. (1982) *Biochemistry,* in press.
13. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132.
14. Engelman, D. M., Henderson, R., McLachlan, A. D. & Wallace, B. A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 2023–2027.
15. Goldkorn, T., Rimon, G. & Kaback, H. R. (1981) *Abstracts of the VII International Congress of Biophysics and III Pan-American Biochemistry Congress, Mexico City* (Congress Center of the Mexican Social Security Institute, Mexico City, Mexico), p. 249.
16. Robertson, D. E., Kaczorowski, G. J., Garcia, M.-L. & Kaback, H. R. (1980) *Biochemistry* **19,** 5692–5702.
17. Garcia, M.-L., Patel, L., Padan, E. & Kaback, H. R. (1982) *Biochemistry,* in press.
18. Goldkorn, T., Rimon, G., Kempner, E. & Kaback, H. R. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41,** 1415 (abstr. 6692).
19. Mieschendahl, M., Büchel, D. E., Bocklage, H. & Müller-Hill, B. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7652–7656.
20. Galfre, G. & Milstein, C. (1981) *Methods Enzymol.* **73,** 3–46.
21. Fazekas de St. Groth, S. & Scheidegger, D. (1980) *J. Immunol. Methods* **35,** 1–28.
22. Littlefield, J. W. (1964) *Science* **145,** 709–710.
23. Nowinski, R. C., Lostrom, M. E., Tam, M. R. & Burnette, W. N. (1979) *Virology* **93,** 111–126.
24. Ey, P. D., Prowse, S. J. & Jenkin, C. R. (1979) *Immunochemistry* **15,** 429–436.
25. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
26. Bowen, B., Steinberg, J., Laemmli, U. K. & Weintraub, H. (1980) *Nucleic Acids Res.* **8,** 1–20.
27. Burnette, W. N. (1981) *Anal. Biochem.* **112,** 195–203.
28. Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Fürst, M., Aichele, G., Wilhelm, U. & Overath, P. (1980) *Eur. J. Biochem.* **108,** 223–231.
29. Kaback, H. R. (1971) *Methods Enzymol.* **22,** 99–120.
30. Short, S. A., Kaback, H. R. & Kohn, L. D. (1975) *J. Biol. Chem.* **250,** 4291–4296.
31. Kaback, H. R. (1974) *Methods Enzymol.* **31,** 698–704.
32. Lowry, O. H., Rosebrough, N. J., Farr, A. J. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
33. Kaczorowski, G. J., Kohn, L. D. & Kaback, H. R. (1978) *Methods Enzymol.* **53,** 519–527.
34. Patel, L., Garcia, M.-L. & Kaback, H. R. (1982) *Biochemistry,* in press.
35. Ramos, S., Schuldiner, S. & Kaback, H. R. (1979) *Methods Enzymol.* **55,** 680–688.
36. Felle, H., Porter, J. S., Slayman, C. L. & Kaback, H. R. (1980) *Biochemistry* **19,** 3585–3590.
37. Rudnick, G., Schuldiner, S. & Kaback, H. R. (1976) *Biochemistry* **15,** 5126–5131.