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Novel S49 Lymphoma Variants with Aberrant Cyclic AMP Metabolism

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SUMMARY

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S49 mouse lymphoma cells resistant to killing by cholera toxin (but sensitive to N^6,$O^{2'}$-dibutyryl adenosine-3',5'-monophosphate) can be selected in a single step. The transition from cholera toxin sensitivity to resistance is stochastic and occurs at a rate of 1.1×10^{-5} per cell per generation. Chemical mutagens increase the frequency of cholera toxin resistance. Screening of independently selected cholera toxin-resistant clones led to discovery of three novel variant phenotypes. Lesions in two of these phenotypes affect the guanine nucleotide regulatory component, called *N*, of adenylate cyclase (EC 4.6.1.1). We assessed the *N* protein in the variants by measuring the ability of membrane extracts to complement *N*-deficient S49 cyc$^-$ membranes *in vitro*, and by radiolabeling peptide subunits of *N* in the presence of cholera toxin and [^{32}P]NAD$^+$. Membranes of one variant phenotype, termed N^{par}, contain about 20% of the *N* activity seen in wild-type (parental) S49 membranes and show reduced radiolabeling catalyzed by cholera toxin. N^{par} membrane extracts partially inhibit wild-type extracts in complementing adenylate cyclase of cyc$^-$ membranes. A second phenotype exhibits cholera toxin-specific radiolabeling of *N* subunits comparable to that seen in wild type, but its *N* protein exhibits very little activity in complementing the defect of cyc$^-$. Resistance to cholera toxin in the third phenotype is associated with normal *N* and adenylate cyclase activities, but extracts of these cells degrade adenosine 3',5'-monophosphate at a rate 4 times faster than wild-type.

INTRODUCTION

Study of variant S49 mouse lymphoma cells has already increased our understanding of hormone-sensitive adenylate cyclase. Because S49 cells are killed by an elevation of intracellular adenosine cyclic AMP, it is possible to select variant clones resistant to cholera toxin or hormonal agonists that stimulate cyclic AMP synthesis. The S49 cyc$^-$ [1] variant was isolated by virtue of its resistance to the cytocidal effect of a β-adrenergic agonist (1). The cyc$^-$ phenotype led to discovery (2) and biochemical characterization (3-6) of a membrane protein, termed *G/F* (3) or *N* (5), that is required for stimulation of cyclic AMP synthesis by hormones, guanine nucleotides, fluoride ion, and cholera toxin. These agents do not stimulate adenylate cyclase in cyc$^-$ membranes, which are deficient in *N* but contain hormone receptors and catalytic adenylate cyclase (2-4). Cyc$^-$ membranes incubated with cholera toxin and [^{32}P]-NAD$^+$ do not show radiolabeling of the 42,000 and 52,000 mol wt peptide subunits of *N* seen in membranes of wild-type (parental) S49 cells (5). Adenylate cyclase in a second S49 variant, called UNC, can be stimulated by guanine nucleotides, fluoride ion and cholera toxin, but not by β-adrenergic amines or prostaglandins (7). The *N* protein of UNC appears functionally "uncoupled" from hormone receptors, and the *N* peptides ADP-ribosylated by cholera toxin in UNC membranes differ in charge from the wild-type peptides (8).

In a search for novel genetic lesions in adenylate cyclase, we investigated clones independently selected for resistance to the cytocidal action of cholera toxin. We found three novel variant phenotypes. Two of these will provide useful tools for elucidating the structure of *N* and its interactions with other components of hormone-sensitive adenylate cyclase. Resistance to cholera toxin in the third variant phenotype appears to result from increased capacity to degrade cyclic AMP. This variant

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[1] The abbreviations used are: cyc$^-$, *N*-deficient S49 variant; *N* or *G/F*, guanine nucleotide regulatory protein of adenylate cyclase; *C*, catalytic unit of adenylate cyclase; UNC, hormone-unresponsive, receptor "uncoupled" S49 variant; dbcAMP, N^6,$O^{2'}$-dibutyryl cyclic AMP; MNNG, *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine; SDS, sodium dodecyl sulfate; GTPγS, guanosine-5'-*O*-(3-thiotriphosphate); Hepes, *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; CT^r, cholera toxin-resistant; PHP, pseudohypoparathyroidism.

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may provide new insight into regulation of cyclic nucleotide phosphodiesterase(s) (EC 3.1.4.17). In the present communication we report the initial characterization of the new variants.

METHODS

Chemicals. All chemicals were obtained from commercial sources as described (6), except for ICR 191 and RO 20-1724, which were gifts from Drs. H. Creech and H. Sheppard, respectively. α-(^{32}P)-ATP and (^{32}P)-NAD$^+$ were purchased from New England Nuclear.

Cells. Clonal sublines of mouse lymphoma line S49.1 were propagated as described (9) in Dulbecco's modified Eagle's medium with 3 g/liter of glucose and supplemented with 10% heat-inactivated horse serum. We used an established wild-type subline (designated 24.3.2) and a previously characterized bromodeoxyuridine-resistant cyc$^-$ cell line, M3B1, which does not complement the original cyc$^-$ line in cell hybrids (10).

Fluctuation analysis. Wild-type cells were plated (200 cells/dish) on soft agar with a fibroblast feeder layer (9). After 10 days, colonies were picked randomly with an Eppendorf pipette and propagated for 8 more days to a population size of 5×10^6 cells. Each of the wild-type subclones (suspended in medium plus serum) was treated for 6 hr with cholera toxin (1 mg/ml) plus RO 20-1724 (0.1 mM), a phosphodiesterase inhibitor, and then plated in soft agar containing cholera toxin (1 μg/ml) plus RO 20-1724. Separate aliquots of each clone were plated in agar containing 0.5 mM dbcAMP. After 12 days, colonies were counted under a microscope. Colonies were picked randomly and propagated to mass culture for characterization.

A reconstruction experiment (data not shown) was performed to test the hypothesis that the selection system kills all wild-type cells but allows all cholera toxin-resistant cells to survive. Toxin-resistant (cyc$^-$) cells were mixed with wild-type cells in known proportions and the mixtures were subjected to cholera toxin selection. The number of cholera toxin-resistant colonies observed (corrected for cloning efficiency) agreed closely with the expected number.

Chemical mutagenesis. A wild-type subclone (divided into three populations) was treated with MNNG (2 μg/ml) for 4 hr, ICR 191 (0.75 μg/ml) for 24 hr, or no mutagen. The cloning efficiency of mutagen-treated populations plated immediately after treatment was approximately 20%. After drug treatment, cells were suspended in conditioned medium and propagated for 1 week for expression of mutations. (Conditioned medium consisted of a 1:1 mixture of normal growth medium, as described above, and of medium in which normal S49 cells had grown to a concentration of 1×10^6 cells/ml before being removed by centrifugation and filtration.) Cholera toxin-resistant colonies were then selected as described above. The number of toxin-resistant colonies was corrected for cloning efficiency and for dbcAMP-resistant colonies, as described in legend to Table 1.

Screening variants. Particulate extracts were prepared from 200-ml cultures ($0.8–1.2 \times 10^6$ cells/ml). The cells were washed once with isotonic buffer (5 mM Hepes, 150 mM NaCl, pH 7.5), resuspended at 4×10^7 cells/ml in membrane buffer (20 mM Hepes, 2 mM $MgCl_2$, 1 mM EDTA, pH 8.0) and placed on ice for 15 min. All further steps were performed at 4°. The cells were sonicated (twice for 5 sec) at a setting of 60 watts with a Biosonik IV sonicator. Sonicates were centrifuged at $200 \times g$ for 3 min, and the supernatant fraction was then centrifuged at $30,000 \times g$ for 20 min. The resulting pellet was resuspended using a Dounce homogenizer into 0.75 ml of membrane buffer containing 10% glycerol, and frozen at −70°.

Cell particulates were treated with no addition, isoproterenol (0.1 mM) plus GTP (0.1 mM), NaF (5 mM), or $MnCl_2$ (10 mM) and adenylate cyclase was measured at 30° using a modification (6) of the procedure of Salomon *et al.* (11). Cyclic AMP synthesis was constant during the 40-min incubation. This procedure provided a semiquantitative assay of the effector-stimulated adenylate cyclase activity in individual clones.

To test for growth inhibition caused by dbcAMP, variant cell populations were exposed for 14 hr to 0.5 mM dbcAMP and their size distributions were determined using a Coulter counter, as described (12). Cells resistant to dbcAMP fail to show the characteristic decrease in size induced by dbcAMP in wild-type S49 populations (12).

Intracellular cyclic AMP. Cellular cyclic AMP was measured by the competitive binding assay of Gilman (13) as described previously (1). Each sample contained lysate from 4×10^5 cells. Samples that contained less than 0.2 pmole of cyclic AMP (the limit of sensitivity) are designated as "≤5" pmole/10^7 cells in Table 2.

Adenylate cyclase. Partially purified S49 membranes, prepared as described previously (14), were incubated in the presence of various effectors under conditions (30°, pH 8.0, 0.3 mM ATP with an ATP-regenerating system) exactly as described previously (15), and cyclic AMP was purified as described (11). Under these conditions, cyclic AMP synthesis was constant during the time of incubation (20 min).

Cholera toxin treatment of membranes. Membranes were incubated (15 min, 30°) with 50 μg/ml of cholera toxin (activated by treatment with dithiothreitol). The membranes were then incubated (15 min, 30°) in a reaction mixture containing 0.1 mM NAD$^+$, 0.3 mM ATP, 0.1 mM GTP, 1 mM thymidine, 5 mM arginine, and 10 mM potassium phosphate (pH 6.8). The membranes were then washed with water, resuspended in membrane buffer, and their adenylate cyclase activity was assayed (as described above) during incubation for 30 min at 30°.

Phosphodiesterase. Whole cell lysates were prepared and their phosphodiesterase activity was measured as described previously (16), using the two-step batch assay of Thompson *et al.* (17), in which cyclic [^{3}H]AMP is converted to 5′-[^{3}H]AMP by cellular phosphodiesterase and then to [^{3}H]adenosine by exogenous 5′-nucleotidase (supplied by *Crotalus atrox* venom). The concentration of substrate (cyclic AMP) was 0.2 μM.

Complementation of cyc$^-$. In vitro complementation of recipient cyc$^-$ membranes was accomplished by adding Lubrol 12A9 extracts ($100,000 \times g$ supernatant fraction) from donor membranes of wild-type and variant clones, using a modification (6, 15) of the method of Ross and Gilman (2).

Complementation experiments using mixtures of extracts of two donor membranes (e.g., wild-type and A3a, in Fig. 1) were performed as follows: Donor membranes (2.5 mg of protein/ml in membrane buffer) were solubilized by adding Lubrol 12A9 to a final concentration of 0.5%. After vigorous mixing and incubation on ice for 30 min, the tubes were centrifuged (100,000 × *g* for 45 min). To destroy catalytic adenylate cyclase activity, the supernatant fractions were incubated at 30° for 10 min and then kept at 4° for 4 hr. The indicated volumes (0–10 μl) of wild-type and/or variant donor extracts were added to test tubes on ice and mixed with heat-inactivated (10 min at 50°) Lubrol-supernatant extracts of cyc⁻ membranes. The volume of cyc⁻ extract was adjusted (between 0 and 10 μl) so that the total amount of detergent and donor membrane protein was the same (equivalent to 10 μl) in all tubes. To each of the tubes containing donor extract were added 40 μl of a solution containing 15 μg of cyc⁻ membrane protein, 0.375 mM α-[^{32}P]ATP (0.3 μCi/tube), isoproterenol (0.1 mM), GTPγS (0.1 mM), and buffer, Mg^{2+}, and an ATP-regenerating system, as described (6, 15). The mixtures were incubated for 40 min and cyclic AMP was purified (11).

Radiolabeling and gel electrophoresis. Membranes (1 mg/ml) were incubated for 30 min with 50 μg/ml of cholera toxin (activated by treatment with dithiothreitol) and 100 μM [^{32}P]NAD^+ (1 Ci/mmole) and other reagents exactly as described (6, 15). Membranes were dissolved by adding SDS and β-mercaptoethanol to final concentrations of 2% and 5% (w/v), respectively, and subjected to discontinuous electrophoresis (18) in 10% polyacrylamide gels, as described (6). Staining, destaining, and autoradiography were performed as described (6).

RESULTS

Does Resistance to Cholera Toxin Result from Mutation?

Fluctuation analysis. To test the hypothesis that resistance to cholera toxin arises by a spontaneous, random event (e.g., a mutation), we performed a fluctuation analysis (19) of the incidence of toxin-resistant cells in populations propagated from 16 independent wild-type S49 clones (Table 1). The mean number of toxin-resistant (and dbcAMP-sensitive) colonies selected from these separate cell populations was much less than the variance in colony number, a result that is consistent with mutation as a cause of toxin resistance. The mutation rate, calculated by the median method (20), is 1.1×10^{-5}/cell per generation. To show that sampling error did not account for the extremely high variance, we selected toxin-resistant colonies from a single wild-type population in 12 replicate plates; the ratio of variance to mean colony number was 0.65.

Effects of mutagens. If a phenotype results from mutation, its frequency should be increased by treating the parental population with chemical mutagens. Wild-type cells were treated with MNNG [a putative base substitution mutagen (21)], ICR 191 [a putative frameshift mutagen (22)], or no mutagen, as described under Methods. MNNG increased the proportion of toxin-resistant colonies from 52 to $342/10^5$ cells, and ICR 191 increased the proportion from 52 to $230/10^5$ cells. These results are consistent with the hypothesis that toxin resistance arises by mutation.

TABLE 1

Fluctuation analysis

A wild-type cell line was cloned in soft agar. Sixteen colonies were picked and each was grown to about 5×10^6 cells. Two hundred cells were plated without selective agents in duplicate to determine cloning efficiency. The rest of the cells were treated with cholera toxin (1 μg/ml) plus RO 20-1724 (0.1 mM) for 6 hr. A known portion from each clone was plated with cholera toxin plus RO 20-1724 in triplicate (1×10^6 cells/dish). Another portion was plated with dbcAMP (0.5 mM) plus RO 20-1724 (0.1 mM) in duplicate (1×10^6 cells/dish). The mean number of dbcAMP-resistant colonies per 1 million cells was subtracted from the mean number of cholera toxin-resistant colonies per 1 million cells to give the number of cholera toxin-resistant, dbcAMP-sensitive colonies per 1 million cells. This number* was corrected for cloning efficiency. Mutation rate was calculated using the median method of Lea and Coulson (20).

Clone	CT^r colonies/10^6 cells	$\bar{X}^a$	dbcAMP^r colonies/10^6 cells	$\bar{X}^a$	Cloning efficiency	$\bar{X}$ CT^r, dbcAMP^r colonies/10 cells*
1	422,437,403	421	0,0	0	0.75	561
2	33,44,35	37	0,0	1	0.85	43
3	27,35,22	28	0,0	0	0.78	36
4	293,275,315	294	140,160	150	0.88	164
5	239,216,200	218	0,0	0	0.86	254
6	102,127,109	113	0,0	0	0.70	161
7	72,71	72	0,0	0	0.87	83
8	16,13,13	14	0,0	0	0.83	17
9	142,127,136	135	0,0	0	0.74	182
10	39,33,29	34	1,1	1	0.81	41
11	22,25,26	24	5,5	5	1.00	19
12	46,56,58	53	0,0	0	0.99	54
13	39,44,45	43	0,0	0	0.59	73
14	3,5,8	5	0,0	0	0.56	9
15	20,13,15	16	0,0	0	0.86	19
16	32,47,47	42	0,0	0	0.70	60

[a] $\bar{X}$, mean number of CT^r, dbcAMP^r colonies/10^6 cells = 111; standard deviation (s) = 140; variance (s^2) = 19,600; $s^2/\bar{X}$ = 177; $d.f.$ = 15; χ^2 = 2,813; $p < 0.005$; mutation rate by the median method = 1.1×10^{-5}/cell per generation.

Screening Variant Clones

Cholera toxin-resistant colonies were picked randomly from agar plates corresponding to the independent wild-type subclones used in the fluctuation analysis. This should enhance the probability of finding different variant phenotypes, because it guarantees that the variants arose during at least 16 different mutational events. In contrast, variants isolated from a single clonal population may often be derived from a single "jackpot" mutation that occurred early in the life of the clone (19).

A total of 28 colonies from the 16 subclones were propagated to mass culture, and adenylate cyclase in particulate extracts of each of these was assayed in the presence of GTP, GTP plus isoproterenol, NaF, or $MnCl_2$, as described under Methods. Mn^{2+} caused an approximate doubling of adenylate cyclase activity in all 28 particulate extracts, as it did in similar extracts of wild-type and cyc⁻ cells. Because Mn^{2+} appears to stimulate activity of the enzyme's catalytic (C) unit directly (3, 10), this result probably indicates that C activity was unaffected in all clones tested.

Of all of the extracts, 23 resembled cyc$^-$ in showing no Mg^{2+}-dependent adenylate cyclase activity in the presence of GTP, isoproterenol plus GTP, or NaF. These are not described in this report. Five clones clearly differed from cyc$^-$: Extracts of four clones exhibited substantial increases in adenylate cyclase activity in the presence of both NaF and isoproterenol plus GTP, while extracts of one clone (H21a) showed an approximate doubling of cyclic AMP synthesis in response to isoproterenol plus GTP, but no response to NaF.

One of the five aberrant clones exhibited resistance to growth inhibition induced by dbcAMP (see Methods). Because this was probably caused by a defect in cyclic AMP-dependent protein kinase (EC 2.7.1.37) (23), this clone was not further analyzed. The other four clones exhibited a wild-type response to dbcAMP, indicating that their cyclic AMP-dependent protein kinase is normal.

Cyclic AMP Accumulation and Synthesis

We first compared cells of the remaining four clones to wild-type and cyc$^-$, with respect to cyclic AMP accumulation in the presence of terbutaline, a *beta*-adrenergic agonist, or cholera toxin plus an inhibitor of cyclic AMP phosphodiesterase (Table 2). Three of the clones showed no response to terbutaline, and one showed a barely detectable rise in cyclic AMP. Cholera toxin, the agent used in selecting these clones, caused a detectable rise in cyclic AMP of three clones, but only to levels 5% to 10% of those measured in toxin-treated wild-type cells. Clone H21a showed no response to either treatment.

For three of the clones, decreased adenylate cyclase activity in partially purified membrane fractions (Table 3) was associated with decreased cyclic AMP measurements in intact cells. Adenylate cyclase in H21a membranes was slightly stimulated by guanine nucleotides and by isoproterenol, a beta-adrenergic agonist, but not at all by NaF. Isoproterenol, guanine nucleotides, and NaF stimulated adenylate cyclase in A3a and T54b membranes, but the activities were only 20%–30% of those observed in wild type.

In contrast, K30a adenylate cyclase activities, unstimulated or in the presence of isoproterenol, guanine nucleotides, and NaF, were comparable to those seen in wild type (Table 3). This raises the possibility that a genetic lesion separate from adenylate cyclase may account for the diminished accumulation of cyclic AMP observed in intact toxin-treated K30a cells (Table 2) and for these cells' resistance to the cytocidal effect of cholera toxin.

TABLE 2

Intracellular cyclic AMP accumulation

Cell populations were treated with terbutaline (0.1 mM), cholera toxin (1 μg/ml) plus RO 20-1724 (0.1 mM), or no addition. Terbutaline treatment was continued for 12 min and cholera toxin treatment for 3.5 hr, since experiments with wild-type cells showed that cyclic AMP peaked at these times for the respective drug treatments. The values represent the mean ± 1 SD of three determinations.

Cell line	Cyclic AMP		
	No addition	Terbutaline	Cholera toxin + RO 20-1724
	pmoles/10^7 cells		
Wild type	<5	594 ± 85	434 ± 40
A3a	<5	14 ± 6	44 ± 2
T54b	<5	<5	23 ± 2
H21a	<5	<5	<5
K30a	<5	<5	20 ± 5
cyc$^-$	<5	<5	<5

TABLE 3

Adenylate cyclase activity in membranes

Adenylate cyclase activity was assayed in the presence of isoproterenol (0.1 mM) plus GTP (0.1 mM), isoproterenol (0.1 mM) plus GTPγS (0.1 mM), GTPγS (0.1 mM) alone, and $MnCl_2$ (10 mM). The mean value is given for adenylate cyclase activity derived from multiple determinations which were within 5% of the mean (except for mean values less than 15 pmoles, for which multiple determinations were within 2 pmoles of the mean). The amount of cyclic AMP synthesis was directly proportional to the amount of protein added and to the time of incubation.

Source of membranes	Cyclic AMP				
	No addition	Isoproterenol + GTP	GTPγS	Isoproterenol + GTPγS	$MnCl_2$
	pmoles/mg protein/min				
Wild type	13	288	279	405	21
A3a	4	51	71	71	11
T54b	5	57	90	124	14
H21a	2	13	12	15	9
K30a	12	280	155	297	20
cyc$^-$	3	3	4	4	9

$MnCl_2$ caused stimulation of adenylate cyclase in all the membranes tested, as in the screening assay.

In our laboratory, maximal adenylate cyclase activities in different membrane preparations can vary by as much as 30%. Accordingly, we measured adenylate cyclase in at least two sets of membranes prepared from each clone. In all cases the results (not shown) agreed with those in Table 3: i.e., H21a membranes showed slight cyclase stimulation by isoproterenol and guanine nucleotides, A3a and T54b membranes exhibited approximately 25% of wild-type activity in response to all the effectors, and K30a adenylate cyclase activity was comparable to wild type.

Treatment of wild-type membranes with cholera toxin and NAD^+ increased GTP-dependent cyclic AMP synthesis, as described previously (24). In A3a and T54b membranes, however, toxin treatment produced about 20% of GTP-dependent cyclic AMP synthesis seen in wild type (Table 4). Toxin treatment partially inhibited stimulation of adenylate cyclase by fluoride ion in wild-type and T54b membranes. After treatment with cholera toxin, H21a membranes showed very slight stimulation of cyclic AMP synthesis in the presence of guanine nucleotides, and cyc$^-$ membranes showed no stimulation of adenylate cyclase in the presence of any of the effectors tested.

N Activity and Radiolabeled Peptides

In vitro complementation. Ross and Gilman (2) and Ross *et al.* (3) first showed that detergent extracts of wild type S49 membranes, rendered devoid of intrinsic adenylate cyclase activity by heating at 37°, can restore to

TABLE 4

Stimulation of adenylate cyclase by cholera toxin

Adenylate cyclase activity was measured in membranes with and without cholera toxin (CT) pretreatment. All additions were 0.1 mM, except NaF = 10 mM. All values are means of duplicate determinations which did not differ by more than 10%.

Membrane	Cyclic AMP after addition of					
	GTP		GTPγS		NaF	
	CT−	CT+	CT−	CT+	CT−	CT+
	pmoles/mg/min					
Wild type	28	318	228	380	457	380
A3a	3	51	24	64	53	51
T54b	6	78	30	98	137	79
H21a	3	7	3	7	4	6
cyc⁻	1	2	1	1	2	1

N-deficient cyc⁻ membranes the capacity to make cyclic AMP in response to isoproterenol, guanine nucleotides, and NaF. To determine whether *N* is the site of the lesion that leads to toxin resistance in the new clones, we added extracts from membranes of each clone to cyc⁻ membranes and measured adenylate cyclase activity (Table 5). As a "blank" we used heat-treated detergent extracts of cyc⁻ membranes, which do not complement cyc⁻ at all.

The results of this *in vitro* complementation assay correlate with direct measurements of adenylate cyclase (Table 3) in membranes. Detergent extracts of H21a membranes are almost as ineffective as those of cyc⁻ in restoring responsiveness of cyc⁻ to NaF or to isoproterenol plus GTPγS (Table 5). A3a and T54b extracts are approximately 20% as effective as wild-type extracts in restoring responsiveness to isoproterenol plus GTPγS and NaF. K30a extracts are as effective as wild type.

One possible lesion of A3a and T54b membranes is that they contain quantitatively diminished but functionally normal *N*. If so, increasing amounts of membrane extract from these variant membranes should be able to complement cyc⁻ as well as smaller amounts of wild-type extract. The results of an experiment comparing different amounts of wild-type and A3a extracts (Fig. 1) suggest that this is not the case. Increasing amounts of wild-type extract appeared not quite to saturate the capacity of a fixed amount of cyc⁻ membranes to make cyclic AMP in response to isoproterenol plus GTPγS; in contrast, adenylate cyclase activity in mixtures of cyc⁻ and A3a extracts reached a plateau that was approximately 20% of the highest activity observed with wild type.

The plateau of activity with increasing amounts of A3a extract suggests that these extracts interact abnormally with cyc⁻, in comparison with wild type. If this abnormal interaction were due to deficiency of a second component (distinct from *N*) in A3a extracts, mixtures of wild type and A3a extracts should produce at least additive effects on adenylate cyclase in the complementation assay. Instead, addition of increasing amounts of A3a extract to a constant amount of wild type extract appears to reduce the complementation of cyc⁻ produced by the wild type extract (Fig. 1, - - -). Thus the A3a extract behaves in the complementation assay as if its *N* had diminished intrinsic complementing activity, but was able to inhibit the complementing activity of wild type. An alternative possibility is that A3a extracts contain an inhibitory activity, potentially separable from *N* itself.

A result similar to that in Fig. 1 was obtained using T54b extracts (data not shown). Thus the A3a and T54b variants, which behave similarly also in assays of cyclic

TABLE 5

Reconstitution of cyc⁻ membranes with Lubrol extract from wild-type and variant membranes

Recipient cyc⁻ membranes were reconstituted with the 100,000 × *g* supernatant fraction of detergent extracts of variant subclone membranes or of wild-type membranes in the presence of GTP (0.1 mM) alone, isoproterenol (0.1 mM) plus GTPγS (0.1 mM), or NaF (5 mM). Each assay tube contained 15 μg of cyc⁻ membrane and 10 μl of Lubrol extract from donor membranes (3 mg of protein/ml), and other reagents, as described under Methods. The rate of accumulation of cyclic AMP was constant during the time of incubation. Wild-types A and B membrane were made from the same subline (24.3.2) on 2 different days. Values are means ± 1 SD of three determinations.

Donor membrane	Cyclic AMP		
	GTP	Isoproterenol + GTPγS	NaF
		pmoles/tube	
Wild type A	4 ± 1	169 ± 4	191 ± 6
Wild type B	5 ± 1	217 ± 5	250 ± 36
A3a	1 ± 1	31 ± 1	32 ± 2
T54b	2 ± 1	29 ± 1	52 ± 1
H21a	1 ± 1	4 ± 1	4 ± 1
K30a	4 ± 1	167 ± 6	191 ± 2
cyc⁻	1 ± 1	2 ± 1	2 ± 1

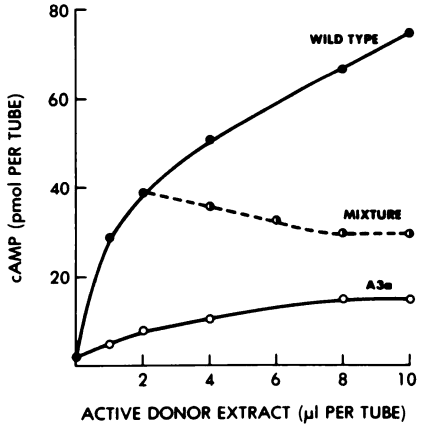

FIG. 1. *Reconstitution of cyc⁻ recipient membranes with wild-type and/or A3a membrane extracts*

Recipient membranes (cyc⁻) were reconstituted with Lubrol extracts (100,000 × *g* supernatant of wild-type membranes (●——●), A3a membranes (○——○), or both wild-type membranes and A3a membranes mixed together (◑- - -◑). In the mixing experiment (◑- - -◑) 2, 4, 6, or 8 μl of A3a extract were added to 2 μl of wild-type extract. Tubes containing less than 10 μl of active extract were supplemented with heat-inactivated extract (see Methods). Each assay tube contained 15 μg of cyc⁻ protein (recipient) and isoproterenol (0.1 mM) plus GTPγS (0.1 mM). Activity is expressed as picomoles of cyclic AMP produced per tube during a 40-min incubation at 30°. Each point represents the mean of three determinations all of which were within 5% of the mean. The mean cyclase activity produced by the addition of 4, 6, or 8 μl of A3a extract to 2 μl of wild-type extract was significantly different from the adenylate cyclase activity seen with 2 μl of wild-type extract alone ($p < 0.05$).

AMP accumulation and adenylate cyclase in membranes, probably bear a similar lesion. We term these variants N^{par} in order to indicate their partial defect in *N* and adenylate cyclase activities.

Radiolabeled N peptides. Incubation of wild-type membranes with cholera toxin and [^{32}P]NAD$^+$ leads to specific radiolabeling of two peptides, of 42,000 and 52,000 mol wt; neither peptide is radiolabeled in cyc$^-$ (5). The smaller of these two peptides is probably a subunit of the *N* protein, because a toxin-radiolabeled peptide of the same molecular weight co-migrates in sucrose gradients and gel exclusion chromatography with *N* activity of another cell type, the human erythrocyte (6).

We treated membranes of the newly isolated S49 variants with toxin and [^{32}P]-NAD$^+$ and subjected them to SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 2). K30a membranes exhibited radiolabeling, of both the 42,000 and 52,000 mol wt peptides, comparable in intensity to wild type. The 52,000 mol wt band was not labeled in the N^{par} membranes, whereas the 42,000 mol wt band showed slight but detectable labeling. These results are in harmony with results in Tables 3 and 4 and Fig. 1: K30a membranes have adenylate cyclase and *N* activities similar to those of wild type, and therefore should show toxin-dependent radiolabeling similar to wild type as well; the N^{par} variants exhibit diminished adenylate cyclase and *N* activities, and thus it is not surprising that they should show diminished radiolabeling of peptide subunits of *N*.

This experiment produced one surprising result: H21a membranes, which possess very little stimulable adenylate cyclase activity and no *N* activity capable of complementing cyc$^-$, exhibit toxin-dependent radiolabeling of the 42,000 and 52,000 mol wt peptides similar in apparent intensity to that seen in wild type (Fig. 2). The same result was seen with two other sets of membranes prepared from H21a (not shown). We have not attempted direct quantitation of the radioactivity associated with these bands. Visual inspection of autoradiograms from multiple experiments, comparing them to bands that are not toxin-dependent (5) and to Coomassie blue staining of the corresponding gels, fails to reveal any difference between the patterns of H21a and wild type. This result contrasts with the failure of cholera toxin plus NAD$^+$ to stimulate H21a adenylate cyclase (Table 4).

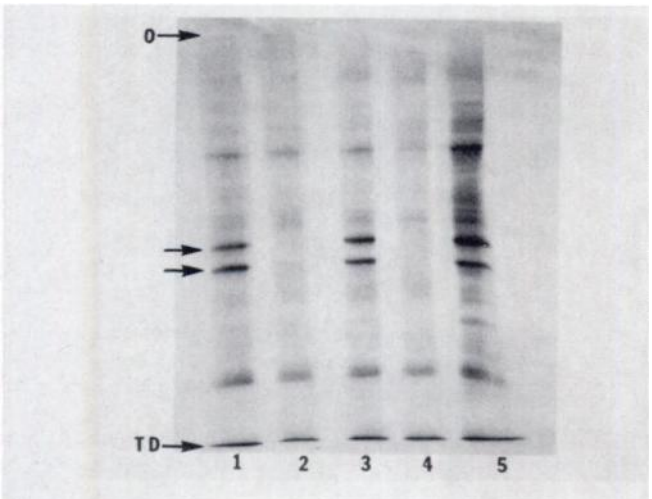

FIG. 2. *Autoradiogram of an SDS slab gel electrophoresis of Lubrol extracts of membranes incubated with [^{32}P]NAD$^+$ and cholera toxin*

1, K30a; *2*, T54b; *3*, H21a; *4*, cyc$^-$; *5*, wild type; *O*, origin; *TD*, position of tracking dye; *top arrow*, M_r 52,000; *bottom arrow*, M_r 42,000. All of the membranes were treated with cholera toxin and radiolabeled as described under Methods. In another experiment, the membranes were incubated with [^{32}P]NAD$^+$ without cholera toxin, and there was no radiolabeling of the 42,000 and 52,000 mol wt peptides (data not shown).

Phosphodiesterase Activity

With respect to wild type, K30a membranes appear completely normal in adenylate cyclase activity, ability of *N* in membrane extracts to complement cyc$^-$ *in vitro*, and toxin-specific radiolabeling (Tables 3 and 5 and Fig. 2). How then can we account for markedly diminished capacity of K30a to accumulate cyclic AMP in intact cells (Table 2) and its ability to survive the cholera toxin selection? One possibility is that K30a is a cyclic AMP transport mutant, able to export cyclic AMP into the extracellular medium at an increased rate. One such S49 variant, already described (25), differs from K30a in being resistant to the cytocidal effect of dbcAMP.

Another possibility is that phosphodiesterase in K30a degrades cyclic AMP more rapidly within the cell, resulting in resistance to agents (e.g., cholera toxin) that act by stimulating cyclic AMP synthesis. This possibility would be consistent with normal sensitivity of K30a cells to dbcAMP, since dbcAMP and its monobutyryl derivative are not significantly degraded by phosphodiesterase.

We tested the second possibility by comparing cyclic AMP phosphodiesterase activity in lysates of K30a and wild-type S49 cells. Lysates of logarithmically growing K30a cells contain 4 times as much phosphodiesterase activity as do wild type (97 versus 24 pmoles of cyclic AMP degraded/30 min per 10^6 cells). Treatment of wild type cells for 4 hr with 1 mM dbcAMP induces a 3-fold increase in phosphodiesterase activity, as previously described (23, 26). The dbcAMP-induced increase in K30a phosphodiesterase activity is less than that seen in wild type, both absolutely and in proportion to the basal activity in untreated cells. (After dbcAMP treatment, wild-type and K30a cell lysates catabolized, respectively, 70 and 122 pmoles of cyclic AMP/30 min per 10^6 cells.)

Because these phosphodiesterase activities were measured at a 0.2 μM substrate concentration, they probably reflect predominantly low K_m phosphodiesterase(s) previously assayed in S49 cells (26). We have not yet explored the many possible mechanisms by which phosphodiesterase activity could be increased in K30a.

DISCUSSION

S49 cells that are resistant to cholera toxin (but sensitive to dbcAMP) can be selected in a single step. On the basis of the fluctuation analysis and the ability of chemical mutagens to increase the frequency of toxin resistance, it is very likely that S49 variants with altered adenylate cyclase arise by mutation. The calculated mu-

tation rate for cholera toxin resistance is high relative to rates determined for many markers studied in somatic cell lines. This may indicate a highly mutable locus, or that S49 cells are functionally haploid for a critical gene product (e.g., a peptide subunit of *N*). Interestingly, mutations that confer glucocorticoid resistance on S49 cells (27) occur at a rate almost as high as that calculated for cholera toxin resistance (3.5 versus 11×10^{-6}/cell per generation), and there is evidence that S49 cells are functionally haploid for the glucocorticoid receptor (28).

Although most of the variant clones isolated in the present study appeared similar to cyc$^-$, we have discovered three novel phenotypes. The phenotypes are sufficiently well defined to allow formulation of working hypotheses regarding their underlying molecular lesions:

Clone H21a. Isoproterenol and guanine nucleotides cause only slight stimulation of adenylate cyclase in H21a membranes and detergent extracts of these membranes are unable to complement the cyc$^-$ lesion, yet cholera toxin catalyzes ADP-ribosylation of the 42,000 and 52,000 mol wt peptides of H21a membranes to an extent similar to that seen in wild type. Because at least the smaller of these peptides is a subunit of the *N* protein (6), this discrepancy must be explained. One possibility is that H21a membranes contain normal *N* proteins but lack an additional component required to complement cyc$^-$. This appears unlikely, because *N* activity migrates in a monodisperse fashion in several physicochemical separation procedures (4, 6) and because of the recently reported purification from liver membranes of a homogeneous protein that can complement all of the defects of cyc$^-$ (29).

To us it appears likely that the H21a lesion will prove to be parallel to but different from that of the UNC phenotype. In the latter variant, a structural change in *N* (detected as a change in electrical charge of cholera toxin substrates) is associated with diminished ability of *N* to interact with hormone receptors (7, 8). A structural lesion in the *N* of H21a membranes may cause it to be functionally "uncoupled" from the catalytic unit of adenylate cyclase, rather than from receptors. It will thus be interesting to determine whether *N* in H21a membranes can mediate guanine nucleotide regulation of receptors for β-adrenergic agonists, as it does in wild type (but not in UNC). As is the case with UNC, the putative *N* lesion in H21a could be due to a structural change in the gene for one of the *N* subunits, or to alteration of an additional cellular activity required for optimal *N* activity.

N^{par} variants. The simplest explanation of the A3a and T54b lesions is that they possess altered *N* proteins which are poor substrates for ADP-ribosylation by cholera toxin and which are incapable of maximally activating the cyclase's catalytic (*C*) unit. The observation that detergent extracts from N^{par} membranes impair activation of cyc$^-$ by wild-type *N* (Fig. 1) suggests that *N* in the N^{par} variants can interact with a saturable component of cyc$^-$, perhaps *C* itself, in a fashion that fails to stimulate cyclic AMP synthesis maximally but prevents normal *N* from doing so. An alternative possibility is that N^{par} membranes contain an inhibitory activity which is physically distinct from *N*. Although not yet ruled out, this alternative explanation appears less likely because it does not account for the reduced ADP-ribosylation of *N* peptides in N^{par} membranes by cholera toxin.

K30a variant. Resistance to the cytocidal and cyclic AMP-elevating actions of cholera toxin in K30a cells is apparently the result of increased cyclic AMP degradation. Potential molecular explanations for the increased phosphodiesterase activity in K30a include increased expression of one or more phosphodiesterase enzymes already present in the wild-type parental cells, an increase in an activator or cofactor of phosphodiesterase, or a decrease in a phosphodiesterase inhibitor. Further characterization of the K30a variant may discriminate among these possibilities, and should increase our understanding of the regulation of phosphodiesterase activity in somatic cells.

Possible relevance to a human disease. Patients with PHP, a heritable endocrine disease, exhibit defective responses to several hormones (e.g., parathormone and thyrotropin) that work by stimulating cyclic AMP synthesis. This laboratory has recently reported (30) that *N* activity is substantially reduced in erythrocytes of most patients with PHP, but not in all. Generalized genetic defects in *N*, other components of adenylate cyclase, or even in phosphodiesterase, could produce the PHP phenotype by causing decreased cyclic AMP accumulation in endocrine target cells. The novel S49 phenotypes reported here, added to the cyc$^-$ and UNC lesions previously defined, provide a challenging view of the many mechanisms by which mutations that affect cyclic AMP metabolism could produce disease in man.

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