CAPPING AND ADENOSINE METABOLISM

Genetic and Pharmacologic Studies*

By JONATHAN BRAUN, FRED S. ROSEN, AND EMIL R. UNANUE

From the Departments of Pathology and Pediatrics, Harvard Medical School; and Department of Medicine, Children's Hospital Medical Center, Boston, Massachusetts 02115

Capping of lymphocyte surface Ig is a striking membrane event in which ligands to surface Ig cause the rapid redistribution of the receptors to one pole of the cell. Several types of evidence indicate that this is a highly organized contractile process: (*a*) it is energy dependent and is sensitive to cytochalasins (1, 2); (*b*) it is associated with the mobilization of intracellular calcium (3) and may be blocked by drugs which interfere with calcium metabolism (4–6); (*c*) it involves the formation of a physical interaction between surface Ig and the underlying microfilament network as judged by immunocytochemical (7, 8), biochemical (9), and biophysical (10) methods; and (*c*) it is accompanied by translatory motion (11). Furthermore, the capacity of surface Ig to associate with and activate the contractile apparatus is a restricted property not shared by other membrane proteins such as the Thy-1 and H-2 alloantigens and the heterogeneous group of surface proteins identified by rabbit anti-murine lymphocyte antibodies (ALG)[1] (12). Capping represents the initial activation phase of a process triggered by surface Ig, which eventually leads to B-cell proliferation and antibody secretion (11).

Recently, methyltransferase reactions have been recognized as an important feature in a wide range of contractile processes, including bacterial (13, 14) and mammalian (15, 16) chemotaxis, lymphocyte-mediated cytolysis (17), endocrine secretion (18), and platelet aggregation (19). These studies generally have depended on pharmacological manipulations that cause an accumulation of *S*-adenosylhomocysteine, a strategy based on the strong inhibition exerted by this metabolite, a product of all adenosylmethionine-dependent methylation reactions, on methyltransferases (20). In eukaryotic cells, *S*-adenosylhomocysteine is catabolized to adenosine and homocysteine by *S*-adenosylhomocysteine hydrolase; thus, by inhibiting the activity of this enzyme, *S*-adenosylhomocysteine accumulates and, consequently, methyltransferase activity falls. Such inhibition usually has been achieved in two ways: (*a*) cells may be fed adenosine and homocysteine, particularly in the presence of agents such as coformycin or *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), which prevent the normal, rapid catabolism of adenosine by inhibiting the enzyme adenosine deaminase (16); (*b*) cells may be treated with 3-deazaadenosine, a potent and specific inhibitor of *S*-adenosylhomocysteine hydrolase. This inhibitor was first characterized

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[1] *Abbreviations used in this paper:* ALG, rabbit anti-murine lymphocyte antibodies; anti-Ig, rabbit anti-mouse immunoglobulin antibodies; anti-θ, anti-Thy-1.2 antiserum; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride; θ alloantigen, Thy-1 alloantigen.

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by Chiang et al. (21) and has been recently applied to studies with murine lymphocytes (17, 22).

In this paper, we have studied capping of lymphocytes in the presence of these two groups of inhibitors and in patients with a hereditary deficiency in adenosine deaminase. We have also tested the effects of the inhibitors on a contractile event directly triggered by the calcium ionophore A-23187. Our results suggest that surface Ig capping requires a methylation reaction which may serve as a triggering step that precedes microfilament activation.

Materials and Methods

Preparation of Lymphocytes. Murine lymphocytes were obtained from 2- to 3-mo-old A/St mice (West Seneca Laboratories, Buffalo, N. Y.) using Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) -Hypaque (Winthrop Laboratories, New York)-purified spleen cells. In some cases, thymocytes were also studied; these were teased from the thymus and used without further purification.

Human blood lymphocytes were obtained from normal volunteers, using dextran sedimentation and Ficoll-Hypaque centrifugation (23). Blood lymphocytes from two patients with combined immunodeficiency resulting from adenosine deaminase deficiency were also studied; the case histories and related biochemical and functional studies have been reported elsewhere (24). These patients were graciously made available to us by Dr. Arye Rubinstein (Albert Einstein School of Medicine, Bronx, N. Y.). Lymphocytes were also studied in two other patients with adenosine deaminase deficiency who had undergone bone marrow transplantation from normal sibling donors 8 yr previously ([25], cases 1 and 2).

All cell suspensions were >90% viable, as judged by exclusion of trypan blue.

Reagents

MEDIA. All studies were performed in Hanks' balanced salt solution supplemented with 10 mM Hepes and either 0.5% bovine serum albumin (for murine lymphocytes) or 2% fetal calf serum (for human lymphocytes).

ANTISERA. Rabbit anti-mouse immunoglobulin antibodies (anti-Ig), purified by ion-exchange chromatography and fluorescein conjugated, were characterized previously (12). Rabbit anti-human IgD and IgM were fluorescein-conjugated Fab'_2 fragments of purified IgG (23) and used for cell staining as a 1:1 mixture. ALG, purified by precipitation in 50% and 40% saturated ammonium sulphate, have been described (3). The anti-Thy-1.2 antiserum (anti-θ) was prepared by immunization of AKR mice with CBA thymocytes. This reagent has been described (12). The fluorescein-conjugated goat anti-rabbit IgG antibodies were an IgG from a goat hyperimmunized to rabbit Ig. The fluorescein:protein ratio was 8.4:1.

The conditions required to induce capping have been described previously and varied according to the type of surface macromolecule: surface Ig, which caps by apparently interacting with the contractile system, redistributes rapidly following interaction with antibody; ALG determinants and the Thy-1 allantigen (θ alloantigen) appear to cap simply by forming large cross-linked complexes, redistribute slowly and only in a fraction of cells, and require multiple layers of antibody to do so (that is, the specific ligand and an antibody to it) (11, 12). Thus, for optimal cap formation, we proceeded as follows: (*a*) surface Ig: $5–10 \times 10^6$ cells were incubated with fluorescein anti-Ig (100 μg per ml) for 30 min on ice and rinsed three times in medium at 0–4°C to remove the unbound ligand. Cap formation generally was induced by incubation at 20°C rather than at 37°C to slow the capping process and to allow a more accurate measurement of this rapid event (4). After 40 min, most cells were capped under these conditions; (*b*) ALG: 5×10^6 cells were stained with ALG (200 μg per ml) for 30 min on ice and rinsed with cold medium. Then, the cells were stained with fluorescein-conjugated goat anti-rabbit antibodies (50 μg per ml) for 30 min on ice and again rinsed with cold medium. Cap formation was induced by incubation at 37°C, yielding ~ 50% capping after prolonged incubation; (*c*) θ alloantigen: $5–10 \times 10^6$ cells were stained with anti-θ (10% dilution) for 30 min on ice and rinsed in cold medium. Then, cells were stained with fluorescein anti-Ig as described above. Cap formation was induced by incubation at 37°C, yielding ~ 50% capping.

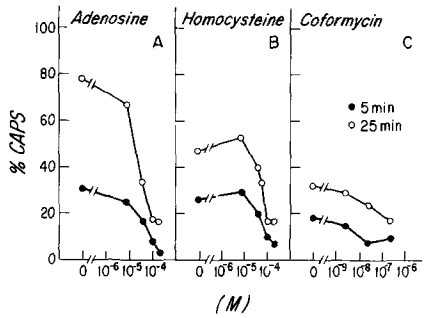

FIG. 1. Murine lymphocytes were preincubated at 37°C for 60 min with inhibitors, then chilled, stained with anti-Ig, and incubated for 5 min (●) or 25 min (○) at 20°C to induce capping of surface Ig. (A) Homocysteine (100 μM), coformycin (0.2 μM), adenosine (as indicated); (B) coformycin (0.2 μM), adenosine (100 μM), homocysteine (as indicated); (C) homocysteine (100 μM), adenosine (100 μM), coformycin (as indicated).

DRUGS. Adenosine and L-homocysteine thiolactone were purchased from Sigma Chemical Co., St. Louis, Mo. EHNA was purchased from the Burroughs Wellcome Co. (Research Triangle Park, N. C.). 3-Deazaadenosine was a generous gift from Dr. Gerald Wolberg of the Wellcome Research Laboratories (Research Triangle Park, N. C.). A-23187 was a gift from Eli Lilly and Company (Indianapolis, Ind.). Coformycin was a gift from Dr. H. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan).

Experimental Protocol. The effect of the inhibitors on cap formation was tested by preincubating 5×10^6 cells per 0.5 ml with various agents for 60 min at 37°C. The cells then were pelleted and incubated with appropriate antibodies at 0–4°C. Finally, the cells were returned to the original volume of medium and drug concentration, incubated for various times to promote capping, and fixed with an equal volume of 2% paraformaldehyde to terminate the reaction.

Cells were scored using a Leitz Ortholux II microscope with epi-illumination (E. Lietz, Inc., Rockleigh, N. J.). Cells were considered capped when the fluorescein label was limited to less than one-half of the cell surface. 200 or more cells were scored for each determination. Experiments on the patients with the adenosine deaminase deficiency were performed on only one occasion. Otherwise, experiments were performed on at least three different occasions with the same results.

Results

Effect of Inhibitors of Methylation on Cap Formation. The effect of inhibitors was tested on capping of surface Ig by preincubating murine or human lymphocytes with various concentrations of adenosine, homocysteine, and coformycin. Fig. 1 shows results using murine lymphocytes. Separately, each of these agents had no effect on capping in the concentration range of 1–500 μM for adenosine and homocysteine and 1 nM–2 μM for coformycin. However, in combination, they inhibited capping in a dose-dependent fashion. Comparable results were found when coformycin was replaced by EHNA: in the presence of EHNA (10 μM) and homocysteine (100 μM), adenosine inhibited capping by 20 and 100% at concentrations of 10 and 100 μM, respectively. Similar results were obtained with human lymphocytes. Fig. 2 illustrates a representative experiment on lymphocytes from a normal human volunteer. As before, each agent separately had little effect on capping; also, in combinations of two agents, little inhibition occurred. However, a combination of all three agents totally abolished capping.

Deazaadenosine caused a dose-dependent inhibition of cap formation, and this was potentiated 10-fold in the presence of 100 μM homocysteine (Fig. 3). These concen-

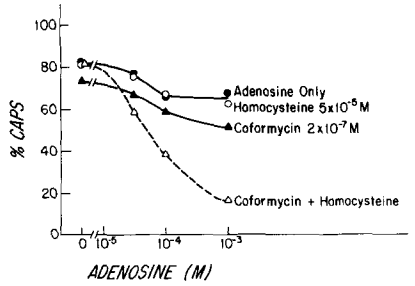

FIG. 2. Human blood lymphocytes from a normal volunteer were preincubated for 60 min at 37°C, then chilled, stained for surface Ig, and incubated for 10 minutes at 37°C to induce capping. Adenosine only: (●); adenosine and homocysteine (50 μM): (○); adenosine and coformycin (0.2 μM): (▲); adenosine, homocysteine (50 μM), and coformycin (0.2 μM): (△).

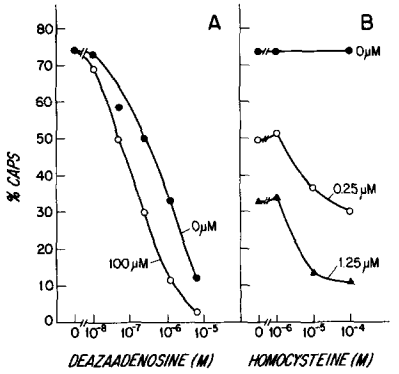

FIG. 3. Murine lymphocytes were preincubated at 37°C for 60 min with deazaadenosine and homocysteine, then chilled, stained with anti-Ig, and allowed to cap at 20°C for 20 min. (A) the dose response to deazaadenosine in the presence of homocysteine at 0 μM (●) or at 100 μM (○); (B) dose response to homocysteine in the presence of deazaadenosine at 0 μM (●), 0.25 μM (○), or 1.25 μM (▲).

tration ranges were comparable to those required for inhibition of lymphocyte-mediated cytolysis (17) and chemotaxis in monocytes (16) and in neutrophils (22). They also corresponded to the range required for the inhibition of methylation in lymphocytes when measured by direct biochemical methods (17, 22).

These initial results demonstrated that both sets of agents were potent inhibitors of surface Ig capping but raised two issues: (*a*) the drug effects could have involved a cell process not directly related to contractile mechanism; (*b*) the perturbation of adenosine metabolism induced by these drugs could have inhibited capping through a mechanism different from methylation (26).

To test for the specificity of these agents for the contractile process involved in capping of surface Ig, we examined, for comparison, the capping of the θ alloantigen and ALG determinants. This class of surface macromolecule caps in an energy-dependent fashion (11) but by a mechanism apparently unassociated with the contractile apparatus (12). (The studies were done only in the mouse because of the more extensive experience with various plasma membrane proteins in this species.) In Fig. 4, concentrations of adenosine, homocysteine, and coformycin that completely abolished capping of surface Ig had little effect on the relatively inefficient capping of the θ alloantigen and ALG determinants. Similarly, concentrations of deazaaden-

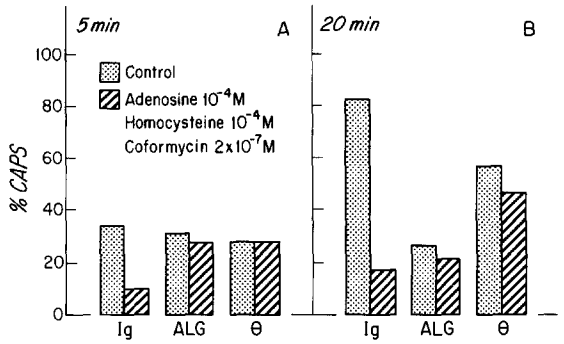

FIG. 4. Murine lymphocytes were preincubated at 37°C for 60 min with inhibitors, then chilled, stained with appropriate reagents, and capped under optimal conditions to promote capping (Materials and Methods). (A) capping after 5 min; (B) capping after 25 min. Note the relative insensitivity of *θ* alloantigen and ALG determinants capping to inhibitors.

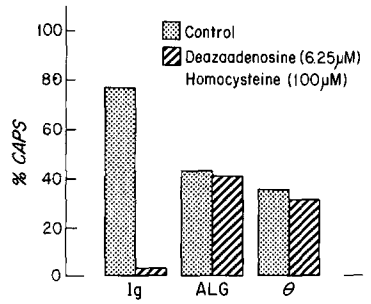

FIG. 5. Murine lymphocytes were treated with inhibitors and capped for 25 min as in Fig. 4.

osine and homocysteine, which well exceeded the levels necessary to block surface Ig capping, had no effect on capping of the other class of surface macromolecules (Fig. 5).

In other experiments, the effects of ethionine and amethopterin were tested on cap formation. However, these agents were highly toxic at inhibitory concentrations (>1 mM) and were deemed unsuitable for further study.

Effect of Inhibitors of A-23187-induced Cap Disruption. The inhibition of Ig capping suggested that the inhibitors might be exerting their effect on the cells' contractile apparatus. This possibility was tested using A-23187-induced cap disruption. In cells which have already formed caps, exposure to A-23187, a calcium ionophore, causes the caps to reverse in a process requiring extracellular calcium and metabolic energy (4) and associated with remarkable arrays of hypercontracted microfilaments (27). On the basis of these observations, A-23187-induced cap disruption is believed to represent a direct and global activation of the microfilaments. Therefore, cap disruption was used to test for a direct effect of the inhibitors on contractile activation. Cells were incubated with anti-Ig at 20°C for 20 min to allow cap formation, treated with drugs for an additional 20 min, and finally treated with A-23187 for 10 min to produce cap disruption (Table I). As expected, deazaadenosine and homocysteine blocked the additional cap formation which occurred between 20 and 50 min (64 and 95%, respectively); however, the agents did not protect the cells from cap disruption by A-23187. Thus, these inhibitors did not appear to act directly on contractile

TABLE I

Effect of Deazaadenosine and Homocysteine on A-23187-induced Cap Disruption

Capping time	A-23187	Deazaadenosine		
		0 μM	0.1 μM	1.0 μM
min	*μg per ml*		%	
20	0	64	—	—
50	0	95	65	62
50	0.1	60	43	18
50	1.0	10	7	2

Cells labeled with anti-Ig were incubated for 20 min at 20°C to induce capping. Deazaadenosine and homocysteine (300 μM) were then added, and the cells were incubated for an additional 20 min. A-23187 was added for the last 10 min to disrupt the formed caps. Values represent percent caps among Ig-positive cells. Note that deazaadenosine and homocysteine blocked the additional cap formation that occurred between 20 and 50 min but did not prevent cap disruption induced by A-23187.

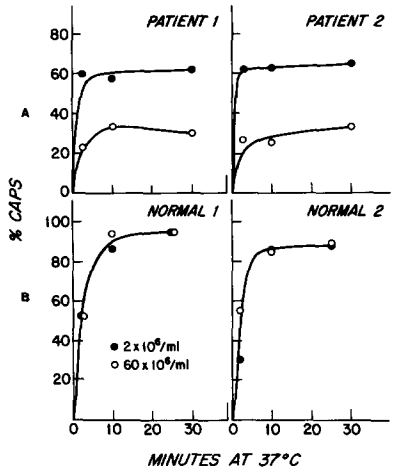

FIG. 6. Human blood lymphocytes were incubated for 120 min at 37°C at a cell density of 2×10^6 per ml (●) or 6×10^7 per ml (○). Cells were then incubated for surface Ig and incubated for various times to promote capping. (A) Results from two different patients with hereditary deficiency in adenosine deaminase. In both groups, the incubation with anti-Ig antibodies and the capping stage were carried out at the same cell concentration (10^6 per ml). (B) Results from two representative normal children.

activation but rather at some point preceding contraction, which was circumvented by the ionophore.

Capping in Patients with a Hereditary Deficiency in Adenosine Deaminase. Studies of capping in the patients with adenosine deaminase deficiency were performed in cells preincubated at two cell densitities, based on the following reasoning: at low cell density, adenosine might be released by cells and be diluted efficiently by the bathing medium, thus dissipating the intracellular levels of adenosine; at high cell density, adenosine would not be diluted efficiently by the medium, and levels of intracellular adenosine would accumulate. In Fig. 6, lymphocytes from normal children capped rapidly and were unaffected by their preincubation at different cell densities; similar

results were found with all five of the other normal subjects tested. In contrast, lymphocytes from the two patients with adenosine deaminase deficiency capped less efficiently. This defect was already evident in cells preincubated at low cell density and quite poorly after the brief incubation at high cell density. Notably, capping was normal in the two patients who previously had undergone successful bone marrow transplantation: >90% capping occurred after 10 min in lymphocytes precultured either at low or high cell density.

Discussion

Our results indicate that putative inhibitors of methylation interfere with capping of surface Ig but not of other surface macromolecules. Experiments with A-23187 suggest that the sensitive step occurs in the signalling phase which precedes contractile activation. Also, a related capping defect appears to exist in patients with a deficiency in adenosine deaminase.

The capacity of surface Ig to interact with the contractile apparatus is an important feature that distinguishes it from the family of surface protein exemplified by θ alloantigen and ALG determinants. Thus, triggering of microfilament activity may be the process affected by the alleged inhibitors of methylation tested in this paper. The effect of these different agents was not simply a result of the inhibition of energy metabolism, because such an occurrence also would have inhibited capping of θ alloantigen and ALG determinants. In addition, the combination of deazaadenosine and homocysteine at these concentrations does not affect the levels of ribonucleotide triphosphates in murine lymphocytes (22). Furthermore, abnormal levels of cyclic nucleotides were unlikely to mediate the drug effects for three reasons: (*a*) increasing cyclic AMP by exposure to permeable cyclic nucleotide analogues, with or without phosphodiesterase inhibitors, does not influence capping of surface Ig (11); (*b*) high doses of concanavalin A inhibit capping as a result of microtubule polymerization that may be induced by excessive intracellular levels of cyclic nucleotides, such inhibition is reversed by colchicine (11). However, colchicine does not protect cells from the inhibition of capping caused by the inhibitors used in this paper (data not shown). (*c*) Deazaadenosine and homocysteine do not affect the levels of cyclic nucleotides in lymphocytes (22).

Studies with A-23187 suggested that the inhibitors acted at a step that precedes contractile activation per se. In this regard, it is interesting to note that, in monocytes, methylation inhibitors appear to block chemotaxis but not phagocytosis (16). As suggested by Pike et al. (16), certain receptors might trigger contraction through a methylation-dependent signal that precedes contraction itself.

In lymphocytes, the earliest known event generated by ligands to surface Ig is the mobilization of intracellular calcium (3). Recently, the effect of deazaadenosine was tested on this ionic event; however, no influence on calcium mobilization could be demonstrated (Unpublished experiments.). The inhibitor-sensitive step thus is separate from, and may be subsequent to, the initial ionic event on capping. The possible relationship between this ionic event and the putative methylation reaction(s) is currently under study.

Studies in the patients with the adenosine deaminase deficiency demonstrated a defect in capping apparently as a result of accumulation of an inhibitory substance,

presumably involving adenosine or a related metabolite like *S*-adenosylhomocysteine and possibly caused by a secondary inhibition of methylation. The possible importance of abnormal methylation in adenosine deaminase deficiency has been emphasized elsewhere (28). However, support of this interpretation awaits direct measurement of methyltransferase activity under these culture conditions and a better understanding of methylation reaction required for capping of surface Ig.

These studies suggest the presence of an early methylation reaction required to trigger surface Ig capping. What might be the nature of this possible methylation step? In bacteria, the substrate for methylation is a class of proteins associated with but distinct from the chemotactic receptors (13, 14). In neutrophils, chemotactic agents stimulate a rise in carboxymethylation that is inhibitable by the appropriate inhibitors; however, this has not been demonstrated in monocytes. In macrophages and in neutrophils, chemotactic agents may induce a change in the levels of methylated lipids (29, 30), although their identities and their relationship to the mechanism sensitive to methylation inhibitors is unclear. It is indeed intriguing to consider the possible role of methylation-induced membrane structural changes (31) on the protein-protein interactions that constitute surface redistribution and cell motility. In this regard, we are investigating methylation reactions in lymphocytes. Aside from confirming that the inhibitors used here do inhibit lipid methylation, we have yet no definite indication that this process is the crucial biochemical event.

The manner in which adenosine deaminase deficiency in man causes the diverse B- and T-cell dysfunctions is not apparent. However, it is likely that a defect in capping may reflect a more general impairment of membrane function, which could be responsible for the inability of the affected cells to properly mature and respond to antigenic stimuli. Finally, the correction of the capping defect in the transplanted patients is of interest. It could indicate that the B lymphocytes were normal and of donor origin; alternatively, normal capping could have resulted from removal of toxic metabolites by the transplanted cells (31).

Summary

Capping of membrane Ig was studied in lymphocytes treated with agents that interfere with adenosine metabolism. Treatment of murine or human B cells with combinations of coformycin, an inhibitor of adenosine deaminase, homocysteine, and adenosine impaired Ig capping. Inhibition of capping was also produced by 3-deazaadenosine, a specific inhibitor of adenosylhomocysteine hydrolase. The inhibitors did not affect capping of the Thy-1 antigen or membrane sites reactive with anti-lymphocyte antibodies. Two patients with a hereditary deficiency in adenosine deaminase had impairment of Ig capping. Such an impairment was not found in lymphocytes of two other patients who had undergone successful bone marrow transplantation.

It is known that the addition of a calcium ionophore results in activation of microfilament function and in disruption of Ig caps. The ionophore effect was not inhibited by the agents mentioned above. Our results suggest that the inhibition of Ig capping during aberrant adenosine metabolism may be caused by a methylation defect preceding the contractile event that produces membrane reorganization.

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