LYMPHOCYTE SURFACE MODULATION AND CYCLIC NUCLEOTIDES
I. Topographic Correlation of Cyclic Adenosine 3':5'-Monophosphate and Immunoglobulin Immunofluorescence during Lymphocyte Capping

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Concepts of plasma membrane structure and function have changed markedly during the past decade (1). The cell surface is no longer considered to be a rigid, static barrier between cytosol and extracellular milieu but rather a dynamic matrix in which cell surface constituents can change their relative and absolute positions (2). Lymphocyte immunoglobulin (Ig) capping is an example of this phenomenon (3, 4). Human peripheral blood B lymphocytes have surface Ig evenly dispersed on the plasma membrane which can be induced to aggregate when cross-linked by polyvalent antisera (5). The formation of local Ig patches is followed by capping which brings the Ig patches to one pole of the cell. Why or how such cross-linking of Ig results in the observed translocation is unknown. However, recent experiments have suggested the involvement of the cell's contractile (microfilaments) (3, 6) and cytoskeletal (microtubule) (7, 8) elements. The data contained in this communication indicate that cyclic adenosine 3':5'-monophosphate (cAMP) may act as one of the signals which regulate cell surface modulation. A dual immunofluorescence technique has been used to demonstrate a topographical correlation between patched and capped surface Ig labeled with rhodamine and specific cAMP immunofluorescence.

Materials and Methods
Surface Immunoglobulin and cAMP Staining. Mononuclear cells were separated from peripheral blood by Ficoll-Isoaque centrifugation. The cells were washed three times in 10% (fetal calf serum (FCS)-RPMI 1640 at room temperature. Viability was assessed by trypan blue exclusion.

The lymphocytes, in a concentration of 1–2 × 10⁶ in 0.05 ml of 10% FCS-RPMI 1640, were incubated with 0.05–0.1 ml of rhodamine-conjugated purified Ig or F(ab')₂ fragments prepared from goat polyvalent anti-human immunoglobulin (9). After a 1 h incubation at 4°C the cells were washed three times with a total of 10 ml of cold 10% FCS-RPMI 1640. Redistribution of cell-bound...
Ig (capping) was effected by incubating lymphocytes stained with the specific fluorochrome-conjugated Ig or F(ab')₂ fragments (azide-free) for 30 min at 37°C.

Localization of cAMP was performed by an indirect immunofluorescent technique. The Ig-capped lymphocytes were fixed on a glass slide (1% paraformaldehyde) overlaid with rabbit anti-cAMP and stained with fluorescein-conjugated goat anti-rabbit Ig (10). In several experiments fixation was omitted and results were unchanged. Ig fractions from nonimmunized rabbits failed to produce significant cAMP staining. Staining the Ig-capped lymphocytes with only fluorescein-conjugated goat anti-rabbit Ig failed to produce fluorescein staining. The specificity of cAMP immunofluorescent technique has been recently reviewed (10).

In experiments utilizing the calcium ionophore A-23187 (a gift from Eli Lilly & Co., Indianapolis, Ind., lot number 361-X17-226) the optimal dose was determined. Incubation of capped lymphocytes with 1.5 μg/ml for 20 min at 21°C reduced the number of capped cells by 75%. After this incubation, lymphocytes were stained for cAMP immunofluorescence. When indicated, sodium azide (10⁻³ M) was incubated with the lymphocytes and polyvalent antisera and patch formation resulted. Lymphocytes were examined by epi-illumination with a Leitz ultraviolet microscope equipped with a mercury arc HBO-200 lamp using BG 38 and 490 nm (fluorescein isothiocynate) exciter filters and a K510 nm barrier filter, and Ploem illuminator.

Results and Discussion

The experiments presented indicate that cAMP immunofluorescence and surface Ig immunofluorescence correlate closely at each stage of cap formation. The initial clustering of Ig, patch formation, was studied in the presence of a metabolic inhibitor, sodium azide, a treatment that blocks subsequent cap formation. Fig. 1 A and B demonstrate a lymphocyte preparation that had been treated with rhodamine-labeled antisera in the presence of 10⁻² M sodium azide and subsequently fixed and stained to detect cAMP immunofluorescence. The microscope field was photographed twice to demonstrate the rhodamine-labeled Ig patches (Fig. 1 A) and the specific cAMP immunofluorescence (Fig. 1 B). The small packets of cAMP immunofluorescence seen (Fig. 1 B) and the larger aggregates seen in later figures were not observed in control lymphocyte populations. The correlation of cAMP immunofluorescence and patch formation was substantiated in three experiments. Thus, cAMP is involved in the earliest stages of the capping process. Fig. 1 C and D show a lymphocyte in the intermediate stage between patch and cap formation. The Ig patches have coalesced into larger aggregates which are migrating toward the future capped pole (1 C). The cAMP immunofluorescence (1 D) is correlated during the Ig migration. The cAMP immunofluorescence seen in Fig. 1 B and D is similar to that observed by Wedner et al. in lymphocytes that have been stimulated by phytohemagglutinin (11).

Fig. 1 E demonstrates a rhodamine-labeled Ig cap, while Fig. 1 F shows the corresponding cAMP immunofluorescence in this lymphocyte. Fig. 2 A shows the rhodamine caps in four contiguous lymphocytes, the cAMP localization in these cells is shown in Fig. 2 B. The association of cAMP immunofluorescence with surface Ig caps has been assessed in lymphocytes from 12 donors. In these experiments, approximately 70% of the Ig caps exhibit concomitant cAMP staining; the range of dual localization varied from 50 to 95%. cAMP immunofluorescence was also observed at the cytoplasmic pole of 5–30% of the non-Ig bearing cells. The significance of this last finding remains to be elucidated. In addition, preliminary results indicate that cGMP is also associated with the fully formed Ig caps (unpublished data).
FIG. 1. The figure shows dual photomicrographs from four types of experiments. The left column reveals the rhodamine-labeled Ig and the right column, the fluorescein-labeled cAMP. A and B show patch formation which occurred when the lymphocytes were treated in the presence of $10^{-2}$ M sodium azide. C and D exhibit an intermediate stage in the capping process. E and F are photomicrographs of a fully capped lymphocyte. G and H demonstrate the results of calcium ionophore treatment of fully capped lymphocytes.

FIG. 2. The correlation between the location of rhodamine-labeled Ig (left) and the specific cAMP antibody labeled with fluorescein (right) in four lymphocytes capped under optimal conditions.

A recent report (12) showed that calcium plus the ionophore A-23187 disrupted mouse lymphocyte caps and prompted experiments to determine if cAMP was involved in cap dissolution. The results show that treatment with calcium and A-23187 at a concentration which did not effect lymphocyte viability also disrupts the Ig cap of human peripheral blood lymphocytes. Cap dissolution appears to be a palindrome of cap formation. The rhodamine Ig aggregates are dispersed by an orderly, time-dependent process in which Ig leaves the cap and...
migrates around the cell periphery. The decapped rhodamine Ig (Fig. 1 G) is still associated with immunofluorescent cAMP (Fig. 1 H) until it becomes totally dispersed.

To show that the involvement of cAMP in cell surface modulation is not restricted to Ig capping, experiments were performed in which the cell surface receptor for the Fc portion of the Ig molecule was capped (13). The procedure involved blocking surface Ig with Fab' fragments and adding exogenous heat-aggregated Ig. Under these conditions the Fc receptor is occupied by the exogenous aggregated Ig and can be capped by addition of polyvalent antisera. Dual immunofluorescent studies during Fc receptor capping have revealed that cAMP is associated with the receptor's localization during patch and cap formation (unpublished results). Thus, cAMP may play an essential role in various types of cell surface modulation.

The utility of the immunofluorescent technique in determining subcellular localization of cAMP in lymphocytes has been demonstrated in several publications. In lymphocytes, different stimuli that are known to increase cAMP levels result in different patterns of cAMP immunofluorescence. Phytohemagglutinin produces packets of cAMP on or near the cell surface. Prostaglandin E1 produces a homogeneous increase in cytoplasmic cAMP staining, while isoproterenol increases intranuclear staining (11). Additionally, lymphocyte contact with latex beads produces increased cAMP immunofluorescence contiguous with the site of contact (10). Implied by these data is the presence of distinct adenylate cyclases in the lymphocyte which respond to specific stimuli with localized increases in cAMP content (14). It is possible that the perturbation of the lymphocyte membrane produced by the Ig cross-linking increases the activity of a local membrane adenylate cyclase.

The present techniques do not allow the assignment of a definitive locus for cAMP action during the capping process. However, recent data suggest that coordinated action of microfilaments and microtubules are involved in cell surface modulation; cAMP could exert an effect by altering the function of these structures. Microfilaments are found in a close proximity to the cell surface and are believed to contain the contractile elements actin, cytoplasmic myosin, and tropomyosin (15). The cytochalasins and increased levels of cytoplasmic calcium ion (produced by the ionophore A-23187) (3, 12) are thought to disrupt coordinated microfilament function and to alter the extent and efficiency of lymphocyte capping. A reasonable interpretation of these results is that coordinated microfilament contraction is initiated by Ig cross-linking and results in Ig translocation. cAMP, through its dependent protein kinase, is known to effect the sensitivity of certain actin-myosin complexes to calcium-triggered contractions (16). Additionally, certain agents that are known to produce lymphocyte capping seem to increase the influx of calcium into the cell (12, 17). The present data and previously cited work can be synthesized into a speculative model. The cross-linking of Ig increases intracellular calcium and produces a localization of cAMP at the sites of Ig aggregation. The cAMP would sensitize the local actin-myosin complex to the calcium signal for contraction and produce coordinated local microfilament contraction.

The participation of microtubules (rigid structures of polymerized tubulin and
associated proteins) in the capping process is more controversial (7, 8, 18). In some instances, microtubular disruption enhances capping; in others it has no effect. However, since rigid microtubule polymerization seems to prevent capping, and the completed cap is known to have microtubules in association with the cap, it is likely that some microtubular rearrangement allows capping to proceed. cAMP-dependent protein kinase is known to phosphorylate certain microtubule-associated proteins (19). The function of these proteins is unknown, but they may participate in coordinated function of microtubules rather than polymerization.

Summary

The cross-linking of human peripheral lymphocyte surface Ig results in an early association of cyclic adenosine 3':5'-monophosphate (cAMP) and the cell surface Ig patches. Examination of the subsequent stages of cap formation reveals the continued relationship of cAMP and the clustered surface Ig. In addition, the generalized influx of calcium produced by the ionophore A-23187 disrupts human lymphocyte caps. During the process of cap dissolution cAMP is still associated with surface Ig. Therefore, it is hypothesized that the localized concentration of cyclic nucleotide and calcium ion regulates the movement of cell surface constituents by coordinating the function of the cell's contractile and structural elements.

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