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Fate of Surface Immunoglobulin During Induction of Lymphocyte Proliferation

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The modulation of immunoglobulin on the surface of rabbit B lymphocytes by goat antibodies with specificity for rabbit surface membrane immunoglobulin or by such goat antibodies covalently linked to Sepharose was studied in relation to the proliferative response to these agents. Although the induction of DNA synthesis was greater in the presence of Sepharose-linked antibody than in the presence of free antibody, modulation of surface membrane immunoglobulin was induced with free but not with Sepharose-linked antibody. Thus, in the presence of free antibody the surface membrane immunoglobulin content of cells was rapidly decreased and remained at a low level throughout the culture period, whereas the surface immunoglobulin content of cells incubated with Sepharose antibody was essentially unaltered. The surface immunoglobulin lost from cells incubated with free goat antibodies reappeared slowly upon further incubation in culture medium devoid of antibody, and such reappearance of rabbit surface membrane immunoglobulin was inhibited by puromycin. Upon culture with Sepharose-linked antibody the surface membrane immunoglobulin content of B cells was unaffected by puromycin. This result was interpreted as indicating that surface membrane immunoglobulin loss followed by reappearance does not occur. Lastly, the linkage of surface membrane immunoglobulin to cytoskeletal elements induced by free antibody was not induced by Sepharose-linked antibody as judged from differences in detergent solubilization characteristics. Possible mechanisms to account for these differences in surface membrane immunoglobulin modulation as they relate to the proliferative response are considered.

Key words: B lymphocytes, proliferative response, surface membrane immunoglobulin, Sepharose linked antiimmunoglobulin, binding assay, immunofluorescence assay, induced internalization, cytoskeleton

Antibodies with specificity for surface membrane immunoglobulin trigger proliferation of rabbit B lymphocytes [1], presumably as a consequence of binding to the same surface membrane immunoglobulin receptors to which antigen ordinarily binds. Although the interaction of antibody with surface membrane immunoglobulin also induces capping and internalization of the antibody: surface membrane immunoglobulin complex [2], a cause-and-effect relationship between internalization of the com-

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plex and induction of proliferation has not been established. Subsequent to initial internalization, the bulk of the surface membrane immunoglobulin reappears when the cells are cultured in the absence of antibody, whereas surface membrane immunoglobulin does not reappear when cells are cultured with antibody [3–5]. While the loss of surface immunoglobulin is complete within 1–2 hr, the induction of DNA synthesis requires the continuous presence of antibody virtually throughout the 48-hr culture period [5–7]. For these reasons it has been postulated that the continuous internalization and reexpression of surface membrane immunoglobulin is required for cell proliferation to occur [5].

Antibody, covalently linked to Sepharose or to polyacrylamide beads, which are larger in size than the lymphocytes, can also induce cell proliferation [8–10]. However, the effect of such particle-linked antibody on internalization of surface membrane immunoglobulin has not been reported. In this study the effect of free and Sepharose-linked antibody was evaluated in regard to both the proliferative response and to the internalization and reappearance of surface membrane immunoglobulin. The results demonstrate that surface membrane immunoglobulin is not internalized in the presence of Sepharose-linked antibody despite the fact that Sepharose-linked antibody induced a greater proliferative response than free antibody.

MATERIALS AND METHODS

Animals

New Zealand rabbits purchased from Dutchland Farms (Denver, PA) weighing approximately 2.0 kg were used in all experiments.

Reagents

Goat antirabbit immunoglobulin and sheep antigoat immunoglobulin were obtained from Cappel Laboratories (Cochranville, PA). Affinity-purified fluorescein-conjugated swine antigoat immunoglobulin and aprotinin (from beef lung) were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). ^{3}H-Thymidine (6.9 Ci/mmol) and Triton X-100 were products of New England Nuclear Corp (Boston, MA). Phenylmethylsulfonyl fluoride was purchased from Sigma Chemical Co. (St. Louis, MO). Puromycin was a product of Calbiochem (San Diego, CA). Cyanogen bromide activated Sepharose 4B was supplied by Pharmacia Fine Chemicals (Piscataway, NJ). Lymphocyte separation medium was from Litton Bionetics (Kensington, MD) and sodium ^{125}iodide (carrier-free) was obtained from Amersham Corp (Arlington Heights, IL). Iodobeads were from Pierce Chemical Co, (Rockford, IL); medium 199 was supplied by Grand Island Biological Co (Grand Island, NY) and fetal calf serum was supplied by M. A. Bioproducts (Walkersville, MD).

Preparation of Sepharose-Linked Antibody

Fifty milligrams of goat antirabbit immunoglobulin (20 mg as antibody) was coupled to 5 g of cyanogen bromide-activated Sepharose 4B by the procedure supplied by the manufacturer. Under these conditions, 95% of the protein was covalently linked to the Sepharose and the antibody content of the beads (4 mg/g Sepharose) was calculated on this basis. ^{125}I-labeled antibody was also coupled to Sepharose by the same procedure.

Iodination of Cell Surface Proteins and of Antibody Preparations

Cells were radioiodinated by lactoperoxidase method of Emerson and Cone [11]. Goat antibody with specificity for rabbit immunoglobulin was iodinated using 10 mg of protein, 1.0 mCi sodium ^{125}I and Iodobeads as described previously [12]. The specific activity of the iodinated protein was 0.1 μCi/μg protein.

Cell Preparation

Single-cell suspensions of spleen were isolated [13] in medium 199 buffered with HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) to pH 7.5 (20 mM) and supplemented with penicillin (35 μg/ml) and fetal calf serum to 10% [14]. When cells were used for surface protein labeling with ^{125}I, splenocyte suspensions were isolated in calcium and magnesium free phosphate buffered saline (Dulbecco and Vogt formulation) at pH 7.3. The cell suspensions were layered over lymphocyte separation medium and centrifuged at 1,500g for 2 min. The cells at the interface were removed and washed three times with medium prior to iodination.

Cell Culture and Proliferation Assay

Two million splenic lymphocytes were cultured in 0.5 ml culture medium for 48 h in 12 × 75-mm capped plastic tubes (Falcon 2058). Antibody and Sepharose-linked antibody were used at the indicated concentrations. All cultures received 5 μCi of ^{3}H-thymidine during the last 24 hr of incubation. Cultures were terminated at 48 hr by the addition of trichloroacetic acid at a final concentration of 5%. The precipitates were washed three times with trichloroacetic acid, solubilized in 2% sodium dodecyl sulfate and counted in Hydrofluor (National Diagnostics, Sommerville, NJ). Data are presented as cpm/10^6 cells and represent the average of triplicate cultures. Variation among triplicates did not exceed $\pm$ 10% of the mean.

Binding of ^{125}I-labeled Antibody

Fifty million cells in 12.5 ml medium were incubated in 250-ml tissue culture flasks (Falcon 3023) with 50 μg/ml antibody or 25 μg/ml Sepharose antibody. At 2, 24, and 48 hr the cells were collected, and, where applicable, Sepharose antibody was removed by passing the cell suspension through a thin layer of glass wool. Recovery of cells was 70–80%. The cells were washed twice and resuspended in fresh medium. Ten million cells in 0.5 ml culture medium were incubated with 30 μg of ^{125}I-labeled antibody for 30 min at 4°C. The cells were then washed four times with medium and counted.

Immunofluorescence

Twenty million cells were incubated with 30 μg of antibody in 0.5 ml medium containing 10 mM sodium azide at 4°C for 30 min, and the cells were layered over 10 ml of calf serum and centrifuged at 500g for 10 min. After washing twice with medium the cells were incubated for 30 min at 4°C with fluorescein-labeled swine antigoat antibody in order to label goat antirabbit antibody associated with surface membrane immunoglobulin. Finally the cells were washed four times with medium, applied to glass slides, and examined by microscopy for fluorescent cells. At least 200 cells were scored for each preparation.

Triton X-100 Extraction and Immunoprecipitation

Fifty million labeled cells were incubated with the indicated concentrations of antibody and Sepharose antibody for 1.0 hr at 4°C. The cells were washed once with medium, the cell pellets extracted with 1.0 ml of 0.5% Triton X-100 containing 1.0 mM phenylmethylsulfonyl fluoride and 100 KIU aprotinin in phosphate-buffered saline at 4°C for 15 min. Lysates were diluted to 0.05% Triton X-100 and centrifuged for 15 min at 1,000g. An aliquot of lysate was incubated with antiserum from a goat immunized with rabbit immunoglobulins and another aliquot of lysate with serum from an unimmunized goat to serve as control. Sheep antiserum with specificity for goat immunoglobulins was added to both aliquots and the sample incubated overnight at 4°C. The resultant specific precipitates were pelleted at 1,000g for 10 min and washed thrice with cold phosphate-buffered saline. The radioactivity in the control was deducted from the radioactivity observed using goat antiserum with specificity for rabbit immunoglobulins. Controls contained about 25% of the radioactivity observed with the antiserum against rabbit immunoglobulin.

Regeneration of Surface Immunoglobulin

Fifty million spleen cells in 12.5 ml medium were incubated with 50 μg/ml of antibody with specificity for surface membrane immunoglobulin for 2.0 hr at 37°C. The cells were pelleted by centrifugation at 500g for 10 min, washed three times with medium and incubated for 16 hr with or without puromycin at 1.0 μg/ml. In other experiments, cells were incubated with or without 25 μg/ml of Sepharose-linked antibody and 1.0 μg/ml of puromycin for 18 hr. At the end of the incubation period Sepharose antibody was removed as described above, and sIg-positive cells were determined on the basis of immunofluorescence.

RESULTS

The effect produced by varying the concentration of antibody and Sepharose-linked antibody having specificity for surface membrane immunoglobulin on the proliferative response of splenic lymphocytes is presented in Figure 1. Detectable stimulation was observed at a Sepharose-linked antibody concentration of 0.1 μg/culture, with maximal stimulation at 10 μg/culture. By contrast, there was no detectable stimulation with free antibody at 5 μg/culture, and maximal stimulation was achieved at 40 μg/culture. At optimal concentrations for both reagents, Sepharose-antibody exhibited a three- to fourfold greater proliferative response than free antibody.

In order to ascertain the stability of the antibody linkage to Sepharose beads, antibody was labeled with ^{125}I prior to coupling to Sepharose. After 48 hr of incubation with cells, the maximal quantity of ^{125}I-labeled antibody released from ^{125}I-labeled Sepharose antibody was 0.23 μg (Table I). This amount was well below the concentration of free antibody required to evoke even a marginal proliferative response (See Fig. 1).

The fate of surface membrane immunoglobulin during stimulation of lymphocytes by free antibody and Sepharose antibody was assessed by immunofluorescence analysis. The data in Figure 2 show that after treatment with free antibody the surface membrane immunoglobulin disappeared within 2 hr and remained at a low level throughout the 48-hr culture period. However, in lymphocytes treated with Sepharose antibody the percentage of surface membrane immunoglobulin-positive cells was

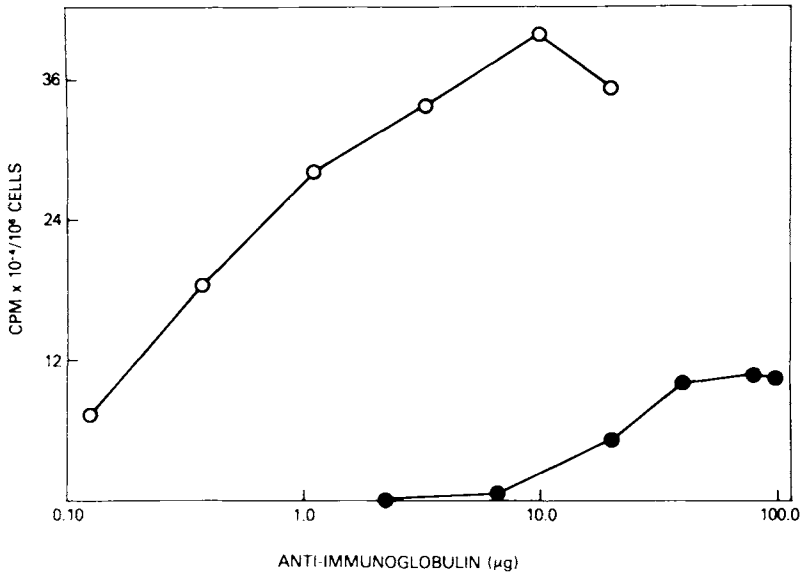

Fig. 1. Effect of antibody (●) and Sepharose-linked antibody (○) on splenic lymphocyte proliferative response. Two million spleen cells were cultured in triplicate for 48 hr in the presence of antibody and Sepharose-linked antibody at the indicated concentrations. The cultures were processed for [^{3}H]-thymidine incorporation as described in Materials and Methods. The values shown are corrected for incorporation in the absence of antibody, which was 27,150 cpm/10^6 cells. Data from a representative experiment are presented.

TABLE I. Release of ^{125}I-Labeled Antibody From Sepharose-Linked Antibody*

	^{125}I-labeled antibody (μg) released at—	
Fraction analyzed	1 hr	48 hr
Cells	0.02	0.014
Culture fluid	0.008	0.22

*Two million splenic lymphocytes were incubated in 0.5 ml of culture medium at 37°C with 20 μg of ^{125}I-labeled antibody linked to Sepharose (4.54×10^5 cpm) for the time indicated. At the end of the incubation period, cells were separated from Sepharose antibody, and the trichloroacetic acid-precipitable radioactivity in the culture fluid and in cells was determined.

essentially equal to that of the control. This observation was further confirmed by determining the binding of ^{125}I-labeled antibody to lymphocytes treated with free and Sepharose-linked antibody (Fig. 3). Cells treated with free antibody exhibited a significant decrease in binding of ^{125}I-labeled antibody. In contrast, the binding by cells treated with Sepharose-antibody was about 80–90% of the controls. This small decrease in binding was shown to be unrelated to the internalization of the surface membrane immunoglobulin. Thus, when Sepharose antibody was removed from cells immediately after its addition, the binding by cells was 80% of the control values.

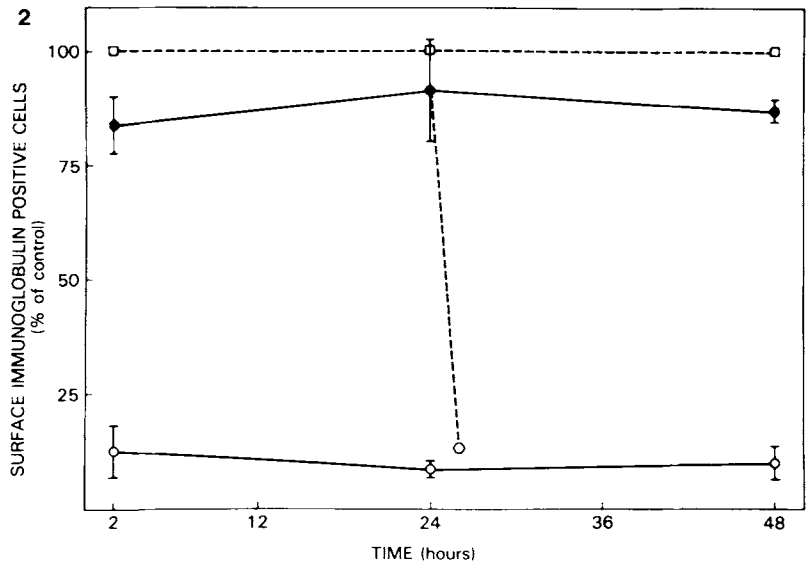

Fig. 2. Effect of antibody and Sepharose-linked antibody on modulation of surface membrane immunoglobulin. Fifty million splenic lymphocytes in 12.5 ml were incubated at 37°C with 50 μg/ml of free antibody or 25 μg/ml of Sepharose-linked antibody for the time period indicated. Cells that were surface immunoglobulin-positive were enumerated using indirect immunofluorescence as described in Materials and Methods. The percentage of surface membrane immunoglobulin-positive cells in the preparation incubated without antibody was 46 ± 1.0, 44 ± 3.0, and 41 ± 2.0 at 2, 24, and 48 hr, respectively and were taken as 100% for each time point. The values presented are the mean ± SEM of three experiments. (□-----□), control; (●-----●), Sepharose-linked antibody; (○-----○), free antibody; (●-----⬡), 2-hr incubation with free antibody added at 24 hr.

Secondly, incubation of cells with Sepharose antibody for 2 hr in an ice bath under conditions in which internalization is precluded yielded a binding that was 78% of the control. It should be noted that the disappearance of surface membrane immunoglobulin upon prior incubation with free antibody is not simply the result of blocking access of fluorescein-labeled or ^{125}I-labeled antibody to surface immunoglobulin. Thus, it was found that less than 15% of antibody initially associated with the cell at 4°C can be detected upon incubation of cells at 37°C to permit internalization [15].

Although Sepharose-linked antibody cannot be internalized because the Sepharose particles are far larger than the relevant cells, one cannot exclude the possibility that surface membrane immunoglobulin is internalized in the presence of Sepharose antibody and rapidly reappears so that the net effect would be the apparent continuous presence of surface membrane immunoglobulin. To test this possibility we used monensin, which has been demonstrated to inhibit the recycling of cell surface receptors subsequent to receptor-mediated internalization [16]. There was no effect on the surface immunoglobulin content of cells incubated with both Sepharose antibody and monensin (data not shown), suggesting that rapid recycling did not occur. Regeneration of surface immunoglobulin is inhibited by agents affecting protein synthesis such as puromycin or cyclohexamide [17, 18]. Experiments showing the effect of puromycin during regeneration of surface membrane immunoglobulin are

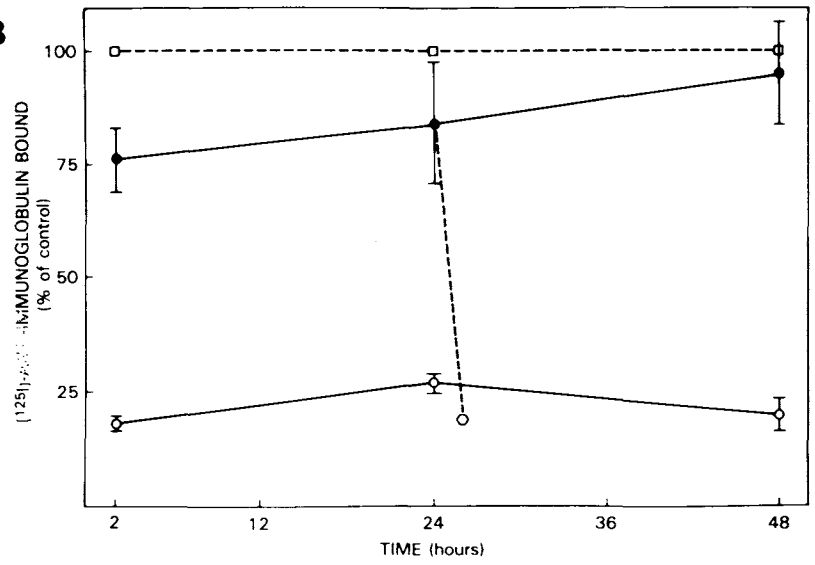

Fig. 3. Binding of ^{125}I-labeled antibody to splenic lymphocytes incubated with antibody and Sepharose-linked antibody. Incubation conditions were as described in the legend to Figure 2. The binding assay is described in Materials and Methods. In the absence of prior incubation with antibody the binding of ^{125}I-labeled antibody ranged from 20 to 30,000 cpm/10^7 cells and was taken as 100% for each time point. The values presented are the mean ± SEM of three experiments. The symbols are as described in Figure 2.

presented in Figure 4. About 60% of the B cells recovered their surface immunoglobulin when cultured for 16 hr in medium free of antibody and this recovery of surface membrane immunoglobulin was significantly inhibited by the presence of puromycin. While puromycin caused a small reduction in surface membrane immunoglobulin-positive cells after 18 hr of culture of cells with Sepharose antibody, the loss of surface membrane immunoglobulin-positive cells was essentially equal to that observed in controls. Since surface membrane immunoglobulin is also shed spontaneously to some extent during cell culture [19, 20], the reduction in surface membrane immunoglobulin-positive cells in the presence of puromycin in both the control and the Sepharose-antibody-containing samples is assumed to be due to this phenomenon.

Treatment of lymphocytes with antibody at 4°C has been shown to induce the association of surface membrane immunoglobulin with cytoskeletal elements, as judged from the insolubility of surface membrane immunoglobulin toward detergent extraction [21]. Subsequent to labeling surface membrane proteins with ^{125}I, cells were incubated with either free antibody or Sepharose antibody and then assayed for detergent-soluble surface membrane immunoglobulin (Table II). Cells treated with free antibody yielded about one-half of the detergent-soluble surface membrane immunoglobulin detectable in control cells, confirming the results of others [21]. However, Sepharose-antibody-treated cells and control cells were indistinguishable in the amount of surface membrane immunoglobulin solubilized with detergent.

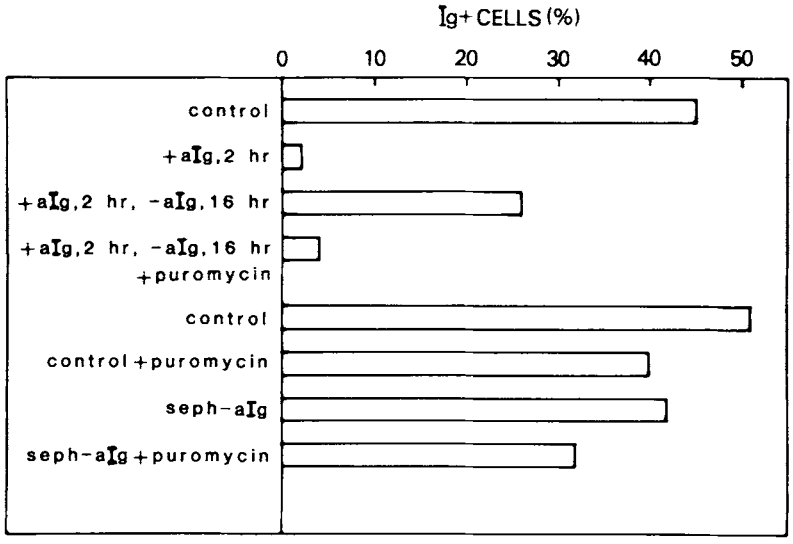

Fig. 4. Effect of puromycin on the appearance of surface immunoglobulin in cells incubated with antibody and Sepharose-linked antibody. Incubation of cells with antibody and enumeration of surface immunoglobulin-positive cells by immunofluorescence was performed as described in Materials and Methods. The values shown represent the mean of two experiments.

TABLE II. Effect of Antibody and Sepharose-Linked Antibody on Triton X-100 Solubilization of Surface Membrane Immunoglobulin*

Cells treated with—	Triton X-100-solubilized antibody (%)
Control (not treated)	100
Antibody	51 ± 5.8
Sepharose antibody	100 ± 4.2

*Fifty million radioiodinated cells were incubated in 12.5 ml of culture medium with 50 μg/ml of antibody or Sepharose-antibody at 4°C for 1.0 hr. The cells were then washed, lysed with 0.5% Triton X-100, and the surface membrane immunoglobulin in the supernatant was assessed by specific precipitation as described in Materials and Methods. Values given are the mean ± SEM of three experiments.

DISCUSSION

Although internalization of surface membrane immunoglobulin was not induced by Sepharose-linked antibody, as the results herein show, the detection of such surface membrane immunoglobulin at any given time may reflect either the retention of the surface molecules in place or the rapid recycling of the molecules subsequent to internalization. Rapid recycling appears to be an unlikely alternative because surface membrane immunoglobulins are down-regulated in the sense that following rapid internalization in the presence of antibody the reappearance of receptors requires many hours rather than minutes. In addition, since rapid recycling apparently does not involve de novo synthesis of receptors, the inhibition of reappearance of surface

membrane immunoglobulin by puromycin would be inconsistent with a rapid recycling mechanism. Finally monensin, which inhibits the reappearance of receptors [16], had no effect on the surface membrane immunoglobulin content when cells were incubated with Sepharose-linked antibody. For these reasons we favor the alternative that surface membrane immunoglobulins are not internalized in the presence of Sepharose-antibody but are retained at the surface.

Two mechanisms of signaling can be envisioned to account for the induction of cell proliferation by mitogens or growth factors. First, the enhanced growth may occur as a result of internalization of the stimulating ligand and/or of the surface membrane receptor. Second, the triggering process may result from transmembrane signaling without an internalization related event or independent of internalization when it does occur. In the case of stimulation of DNA synthesis in response to epidermal growth factor, the evidence has been interpreted by different laboratories to suggest both possible mechanisms [22]. The results obtained herein using Sepharose antibody to trigger lymphocytes indicate that, at the very least, the bulk of the surface membrane immunoglobulin is neither internalized nor becomes associated with the cytoskeleton as a prelude to triggering of proliferation, ie, triggering is apparently independent of such modulation of receptors. The absence of internalization of surface membrane immunoglobulins on cells incubated with Sepharose antibody best fits the view that the triggering of proliferation occurs by transmembrane signaling. A logical extension of this interpretation is that free antibody also stimulates the cells by transmembrane signaling and that the modulation of surface membrane immunoglobulin by free antibody relates to some other cellular need or process.

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