Cross-linking of IgE-Receptor Complexes by Rigid Bivalent Antigens >200 Å in Length Triggers Cellular Degranulation

Patricia M. Kane, David Holowka, and Barbara Baird

Department of Chemistry, Cornell University, Ithaca, New York 14853

Abstract. We have examined the effect of cross-linking IgE–receptor complexes with variable receptor–receptor distances on the transmembrane signaling that leads to degranulation of rat basophilic leukemia cells. Linear polymers of the biotin-binding protein avidin were generated with bis biotin-1,12-diamidododecane, and a dinitrophenyl–biotin conjugate was bound at each end of the polymers to form a series of rigid bivalent haptens of well-defined length. The polymers were fractionated by size with nondenaturing PAGE, electroeluted, and tested for their ability to stimulate degranulation of rat basophilic leukemia cells sensitized with anti-DNP IgE. We found that hexamers of avidin (of length ≥240 Å) were as effective in triggering degranulation as dimers (of length ~80 Å), while the monomeric avidin antigen (of length ~40 Å) elicited a poorer degranulation response from the cells. The mechanism by which aggregation of cell surface receptors can initiate signal transduction is discussed in light of these results.

THE aggregation of cell surface receptors in response to biological stimuli is an important mechanism for initiating signal transduction across cell membranes. In particular, the aggregation of cell surface molecules plays an important role in the immune response; for example, T cell–independent antigens activate B cells by cross-linking surface Ig (Monroe and Cambier, 1983; Dintzis et al., 1983). Perhaps the most widely studied example of this process is the cross-linking of IgE–receptor complexes on mast cells, basophils, and rat basophilic leukemia (RBL)[1] cells, a tumor cell line of mast cell lineage (Seldin et al., 1985). These cells all have cell surface receptors that bind monomeric IgE with high affinity and secretory granules that contain histamine, serotonin, and other mediators of the allergic response. The binding of IgE to its receptor causes no detectable perturbation of the cells, but in response to cross-linking of the IgE receptors by antigens or other means, a complex series of biochemical events is initiated, culminating in the degranulation of these cells (Metzger et al., 1986).

Since the earliest postulation of the importance of cross-linking (Ovary, 1961), the aggregation of IgE receptors on the cell surface has been well-established as the initial requirement for signal transduction leading to degranulation (Metzger, 1983). However, it is still not known what aspects of the cross-linking event are critical for generation of a transmembrane signal. For example, cross-linking could induce contact between receptors, perhaps forming an ion channel or causing a mutual conformational change in the receptors. Alternatively, the simple tethering of receptors to each other, with the orientational constraints and potential for multivalent interactions with other cellular components inherent in this tethering, could initiate signal transduction.

In this study, we have attempted to address the question of whether cross-linked receptors must be brought into contact to trigger degranulation by using a series of rigid bivalent haptens of defined length to aggregate the IgE–receptor complexes. For these experiments, we used linear polymers of the tetravalent, biotin-binding protein, avidin. The polymers were prepared by the addition of bis-biotin-1,12-diamidododecane (bis-biotin) to avidin as described by Green and colleagues (1971, 1975). Dinitrophenyl (DNP) groups, bound to the free biotin binding sites at each end of the linear polymers as a DNP-biotin conjugate, served to cross-link anti-DNP IgE–receptor complexes into linear chains as was shown for unpolymerized avidin antigens by Kane et al. (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). The results described here indicate that directly cross-linked receptors may not need to be brought into contact to stimulate degranulation.

Materials and Methods

Materials

1-DNP-12-biotinamidododecane (DNP-C_{12}-biotin) and bis-biotin were gifts from Dr. N. Michael Green (MRC, Mill Hill, London, UK). Affinity

1. *Abbreviations used in this paper*: bis-biotin, bis-biotin-1,12-diamidododecane; BS^3, bis-sulfosuccinimidyl suberate; DNP, 2,4-dinitrophenyl; DNP-C_{12}-biotin, 1-DNP-12-biotin amidododecane; (DNP-C_{12}-biotin)-avidin, DNP-C_{12}-biotin bound to avidin after dialysis to remove unbound or loosely bound hapten; RBL, rat basophilic leukemia.

purified hen egg white avidin, bissulfosuccinimidyl suberate (BS^3), and dithiobis(sulfosuccinimidylpropionate) were from Pierce Chemical Co. (Rockford, IL) and fluorescamine was from Sigma Chemical Co. (St. Louis, MO). Monoclonal HI.26.82 anti-DNP IgE (Liu et al., 1980) was purified, iodinated, and fluorescein-labeled as described (Holowka and Baird, 1983; Erickson et al., 1986).

Formation of Avidin Polymers

A small volume of 1 mM bis-biotin in 85:15 ethanol/DMSO was added to 1 mg/ml avidin in PBS (50 mM sodium phosphate, 0.15 M NaCl, pH 7.4) with vigorous vortexing. Then 1.5 mol of excess avidin per mole of the initial avidin was added to cap the polymers formed. An optimal dose of 0.75–1.0 bis-biotin/avidin site (four sites per avidin) yielded >50% avidin polymers that were predominately dimers to hexamers.

The ends of the avidin polymers were haptenated in PBS with DNP-C_{12}-biotin using amounts calculated from a titration in which the fluorescence quenching of the avidin tryptophan was monitored (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). A slight excess of DNP-C_{12}-biotin needed for saturation was added as 3–4 small volumes of an ethanol solution, with 15–20 min of mixing between additions. The resulting mixture was then centrifuged (9,000 *g* for 1.5 min), and the supernatant was dialyzed overnight. For polymer preparations that were also cross-linked with BS^3 and fluorescently labeled with fluorescamine, the order of addition to avidin was BS^3, DNP-C_{12}-biotin, then fluorescamine.

Chromatography

In some experiments the polymer mixture in 0.2 M NaCl, 20 mM Hepes, 0.02% NaN_3, pH 7.1, was passed over a column of Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ; 0.95 cm^2 × 77.0 cm; 4.2 ml/h). Molecular mass standards (Bio-Rad Laboratories, Richmond, CA) were run to standardize the column, and the elution volume of a solution of untreated avidin corresponded to a molecular mass of 69 kD according to this standardization.

HPLC gel permeation separations were performed using a Superose 6 column (Pharmacia Fine Chemicals) on an HPLC system (Waters Associates, Milford, MA) equipped with a 280 nm absorbance detector. For preparative runs, 200–500 μg of protein were eluted by isocratic 20 mM sodium phosphate, 0.1 M sodium sulfate, 0.02% NaN_3, pH 7.4 buffer at a flow rate of 0.3 ml/min. Comparison of retention times with those of the molecular mass standards indicated that avidin labeled with fluorescamine and/or BS^3 eluted at the expected time for its molecular mass, while unmodified avidin was retained considerably.

Chemical Modifications of Avidin and Avidin Polymers

For fluorescence modification a 100-fold molar excess of fluorescamine in DMSO was added as three small additions interspersed with 30–60 s of vigorous vortexing to 0.5 mg/ml avidin in 0.1 M sodium borate, 80 mM sodium chloride, pH 8.4–8.5 at ambient temperature (Bohlen et al., 1973), and then the solution was extensively dialyzed. For covalent cross-linking of avidin polymers, equal volumes of 10 mM BS^3 (or dithiobis [sulfosuccinimidyl-propionate]) in PBS and 1 mg/ml avidin in the same buffer were mixed for 3.5 h at 4°C (Staros, 1982), and the reaction was quenched by addition of a 10-fold excess of glycine over BS^3.

PAGE and Electroelution

Avidin monomer and polymers that had not been chemically modified were separated by the nondenaturing cationic electrophoretic system described by Reisfeld et al. (1962), with a 7.5% acrylamide running gel (pH = 4.3) and a 4% acrylamide stacking gel (pH 6.7; 1 mm thick). Samples were diluted or dialyzed into a buffer containing 20% glycerol, 80 mM potassium acetate, pH 6.7, for loading onto the gel. Reaction of the avidin with BS^3 or fluorescamine reversed the mobility of the protein in this PAGE system such that the labeled proteins ran toward the anode rather than the cathode. For these labeled proteins, a nondenaturing anionic electrophoresis system (Clarke, 1964) was used, with running and stacking gels at pH 8.8 and 6.8, respectively. Samples were loaded in 20% glycerol, 0.08 M Tris, pH 6.8. Proteins were detected by staining with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories).

Preparative separations were all performed in the anionic electrophoresis system, and 200–400 μg of protein per lane were loaded on to the gel (1 or 2 mm thick). After electrophoresis the protein bands were detected by their fluorescamine fluorescence under UV light and excised. Pieces of gel were soaked for 5–10 min in electroelution buffer (20 mM Tris-HCl, 5 mM NaCl, 0.2 mM EDTA) then placed in the chamber of an analytical electroeluder (International Biotechnologies, Inc., New Haven, CT). The proteins were electroeluted under a constant current of 50 mA for 90 min and collected in a salt cushion consisting of 3 M sodium acetate, 0.1% gelatin (wt/vol), 15% glycerol (vol/vol), and ~10 μM fluorescein (added to distinguish the salt cushion from the overlying running buffer). Protein concentrations in dialyzed samples were determined from the fluorescamine fluorescence (excitation at 400 nm, emission at 480 nm) by comparison with a known concentration of fluorescamine-labeled avidin that had not been subjected to electrophoresis. DNP concentrations were estimated by assuming 1 DNP bound at each end of the polymers (Kane P., D. Holowka, and B. Baird, manuscript submitted for publication). Material electroeluted from gel pieces containing no avidin did not stimulate degranulation above background, and did not inhibit degranulation triggered by the multivalent antigen, DNP_{23}-bovine serum albumin.

Electron Microscopy

Microscopy was carried out on a Philips 301 electron microscope with 100 kV accelerating voltage. For rotary shadowing, avidin samples were prepared in 60% glycerol (protein concentration = 3 μg/ml) and atomized onto a surface of carbon-coated mica. The samples were then coated with vaporized Pt/Pd for 1.0–1.5 min, and the shadowed material was picked up on a copper grid (300–400 mesh; Telford et al., 1980). For negatively stained preparations, avidin samples (protein concentration = 3–65 μg/ml) were applied by the drop method (Haschemeyer and Myers, 1972) to a carbon film supported on a copper grid (400 mesh) and then stained with 1% uranyl formate.

To assess the distribution of avidin polymer lengths for comparison to the molecular mass distribution provided by PAGE analysis, the lengths of ~575 polymers from an HPLC fraction were measured with a 10× measuring magnifier in negatively stained electron micrographs (472,000×). Those polymers with both dimensions corresponding to <65 Å were designated monomers, based on Green's determination of the 41 × 55 × 55 Å structure (Green et al., 1971). The longer dimension of the designated monomers from the measured polymers corresponded to a length of 59.0 ± 4.6 Å. The shorter dimension of the designated monomer (which should be a statistical average of the 41 and 55 Å lengths) and the longer dimension of the other polymers (which should be some multiple of the 41 Å length) were then plotted as a distribution of polymer lengths. About 12% of the total polymers could not be scored because of poor resolution, and ~4% were not scored because both dimensions were >67 Å, presumably representing the projections of stacked molecules.

Binding Studies

The binding of DNP-C_{12}-biotin-BS^3-avidin to FITC-IgE was measured by the fluorescence quenching method (Erickson et al., 1986) and analyzed as described by Kane et al. (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). The monomer sample used for these studies had been purified by gel permeation HPLC and had 1.70 DNP-C_{12}-biotin molecules per avidin based on its absorbance spectrum.

Cell Experiments

RBL subline 2H3 cells (Barsumian et al., 1981) were grown, harvested, sensitized with IgE, loaded with ^{3}H-5HT, and tested for degranulation as previously described (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). All degranulation assays were carried out at 37°C in the presence of 30% D_2O (Fewtrell and Metzger, 1980). For the in situ reduction experiments shown in Fig. 7, DTT was added to a solution of electroeluted (DNP-C_{12}-biotin)-BS^3-avidin or (DNP-C_{12}-biotin)-DTSSP-avidin to give a final concentration of 2 mM in buffered salt solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 0.1% gelatin, 20 mM Hepes, pH 7.4). The triggering solutions, with and without DTT, were mixed with an equal volume of cell suspension. Inclusion of 1 mM DTT in the buffer caused an increase of <2% in the spontaneous release when cells were incubated for 90 min at 37°C.

Fluorescence microscopy and fluorescence photobleaching recovery measurements were performed as described by Kane et al. (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication) using cells sensitized with FITC-IgE. HPLC fractions from (DNP-C_{12}-biotin)-BS^3-polymer and (DNP-C_{12}-biotin)-BS^3-monomer samples were used in the photobleaching experiments. Analysis by nondenaturing PAGE indicated that the polymer fraction contained only trimers and larger polymers.

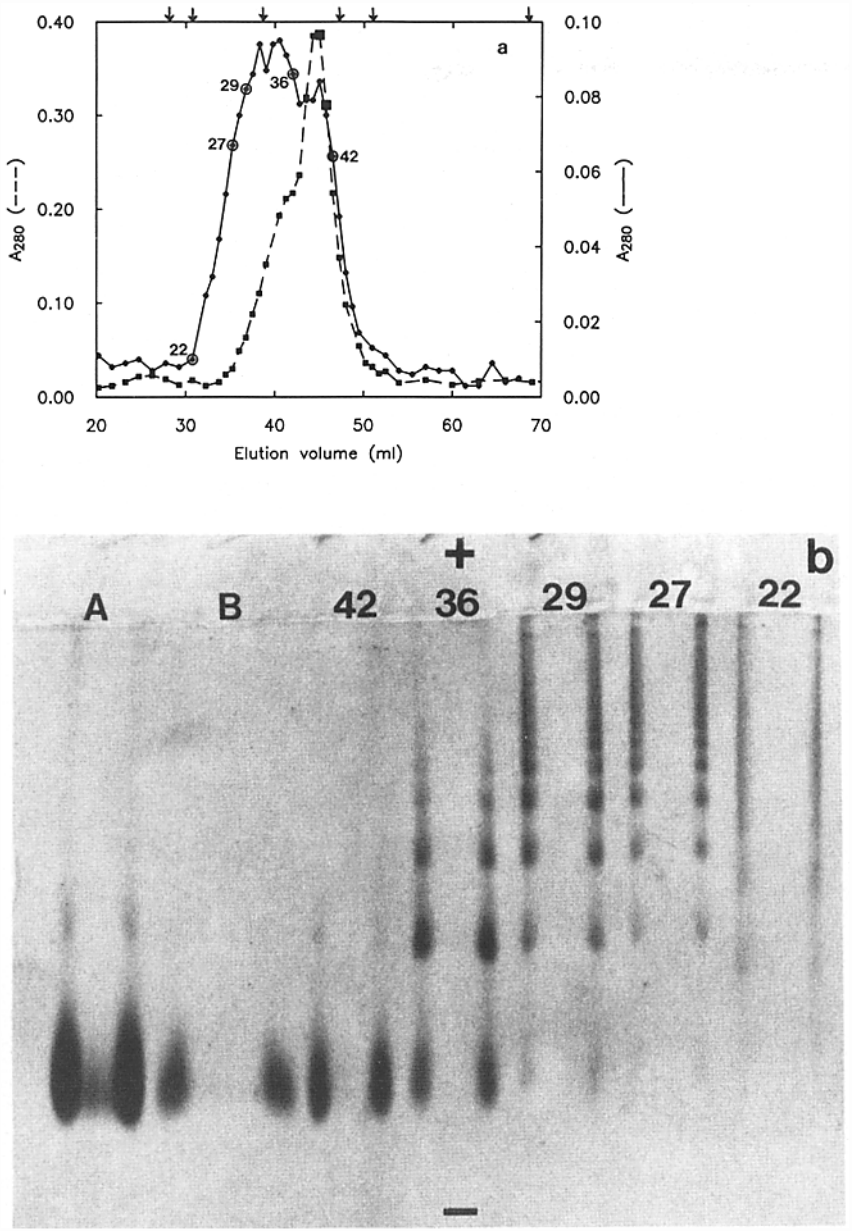


Figure 1. Molecular mass distribution in an avidin polymer preparation determined by gel filtration and nondenaturing PAGE. (*A*) One equivalent of bis-biotin per avidin binding site was mixed with avidin, and this mixture was separated on a Sephacryl S-300 gel filtration column (■). The fractions marked (▣) were combined, excess avidin (2 eq. based on original avidin) was added, and the mixture was rerun on the same column (●). Molecular mass standards are indicated by arrows (from *left*): void volume thyroglobulin (670 kD), γ-globulin (158 kD), ovalbumin (44 kD), myoglobin (17 kD), and vitamin B12 (1.35 kD). (*B*) The fractions indicated (◉ from *A*) on the gel filtration profile were run in the cationic PAGE system described in Materials and Methods. Numbers at the top of the gel refer to fractions in *A*. Lanes *A* and *B* show the migration of avidin monomer and avidin monomer in combination with DNP-C_{12}-biotin, respectively. The separation of bands into two parallel spots that migrate at the edges of each lane is typical for avidin.

Results

Preparation and Purification of Avidin Polymers

In initial experiments avidin polymers were formed by mixing avidin with an excess of bis-biotin, dialyzing away excess unbound ligand, and adding more avidin to cap the polymers, thus ensuring that there were empty biotin binding sites at the ends of the avidin polymers. The distribution of polymers obtained by this procedure can be assessed by gel filtration chromatography, as shown in Fig. 1 *A* (■). After the first incubation of avidin with biotin, predominantly monomer is formed, but there is a substantial shoulder at higher molecular masses. When the second capping step is carried out on monomer fractions, a distribution of sizes is obtained including peaks corresponding to molecular masses for avidin monomer, dimer, and trimer as well as some higher molecular mass material (Fig. 1 *A*; ●). In subsequent experiments, no attempt was made to isolate a monomer fraction after the initial bis-biotin and avidin incubation, and the whole mixture was capped with monomeric avidin. The distribution of avidin polymer sizes in the gel filtration fractions after the capping step can be visualized by nondenaturing PAGE which separates the polymers into discrete bands that appear to migrate according to size (Fig. 1 *B*).

For preparative PAGE, it was necessary to label the avidin molecules with a fluorescent marker before electrophoresis so that bands could be excised for electroelution without the need for fixing and staining. After testing several different fluorescent modifying reagents, including fluorescein isothiocyanate, tetramethyl rhodamine isothiocyanate, lucifer yellow vs, and mansyl chloride, none of which was satisfactory, we found that fluorescamine reacted with avidin and its polymers very efficiently to yield a fluorescent derivative that was visible on UV-illuminated polyacrylamide gels in

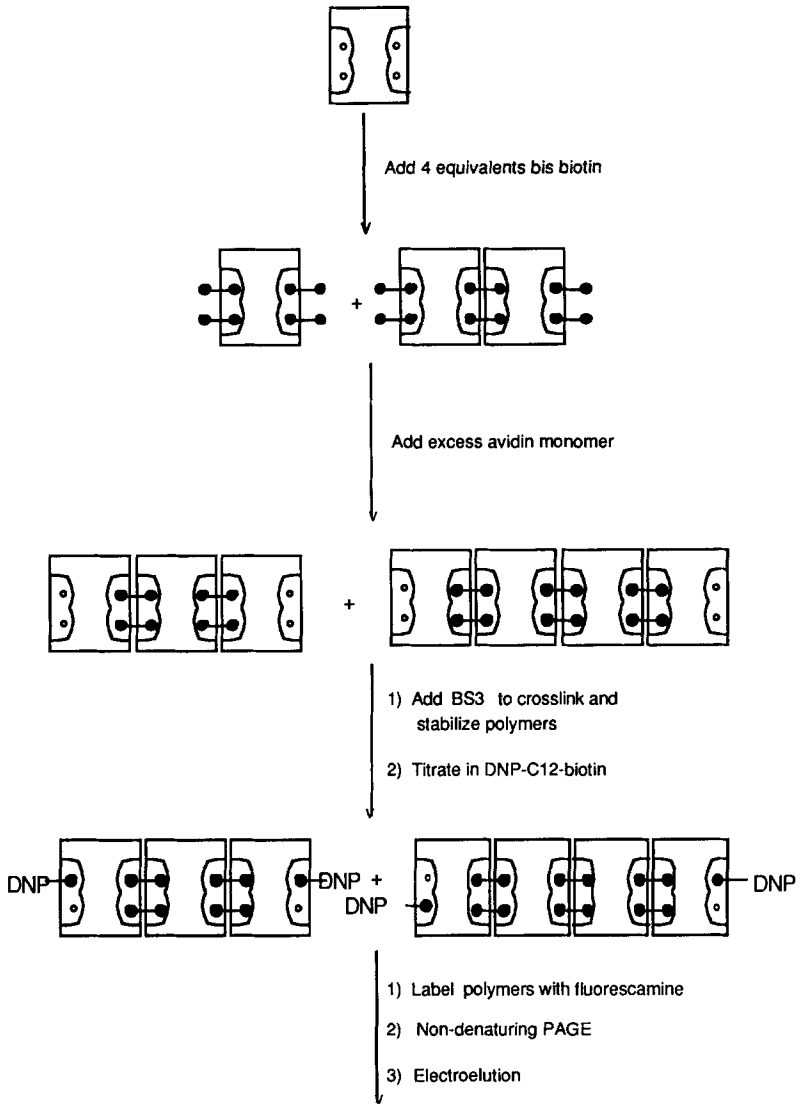


Figure 2. Protocol for preparation of avidin polymers. One 41 × 55-Å face of the avidin monomer, cut away to reveal the four biotin binding sites, is shown. Empty biotin binding sites (○), bis-biotin-diaminidodecane (●——●), and DNP-C_{12}-biotin (●——DNP) are shown.

submicrogram quantities per band. Since fluorescamine reaction with amino groups changes the net charge of the avidin protein, an anionic electrophoresis system was used.

The fluorescamine modification also appeared to destabilize the avidin polymers, and to preserve the original polymer distribution, covalent intramolecular cross-linking before fluorescamine modification was employed. Of several cross-linking reagents tested, including 3,3′-dimethyl suberimidate and difluorodinitrobenzene, BS^3 was found to provide the optimal balance of polymer stabilization and very little intermolecular cross-linking. The order of addition of BS^3, fluorescamine, and DNP-C_{12}-biotin to the avidin polymers proved to be important. It was necessary to titrate DNP-C_{12}-biotin into the biotin binding sites after cross-linking with BS^3 since the DNP ligand appeared to promote intermolecular cross-linking. Modification of the avidin polymers with BS^3 did not appear to change the affinity or stoichiometry of DNP-C_{12}-biotin binding to avidin. Although modification with BS^3 did decrease the amount of fluorescamine label that could be incorporated, it did not prevent the polymers from being visualized after PAGE. In the final preparations, avidin monomer and polymers were cross-linked with BS^3, titrated with DNP-C_{12}-biotin, then labeled with fluorescamine. A schematic of this protocol for avidin polymer preparation is shown in Fig. 2.

Structural Characterization of Avidin Polymers

The avidin polymers could be visualized directly by electron microscopy. In agreement with the observations of Green et al. (1971), they appear as predominantly linear, rigid rods in both rotary-shadowed and negatively stained electron micrographs (Figs. 3 and 4 *A*, respectively), and some images of the polymers reveal a repeat unit that corresponds approximately to the length of an avidin monomer (Fig. 3). Examination of several different high magnification micrographs of various avidin polymer preparations showed no evidence of molecules that were clearly branched due to binding of two

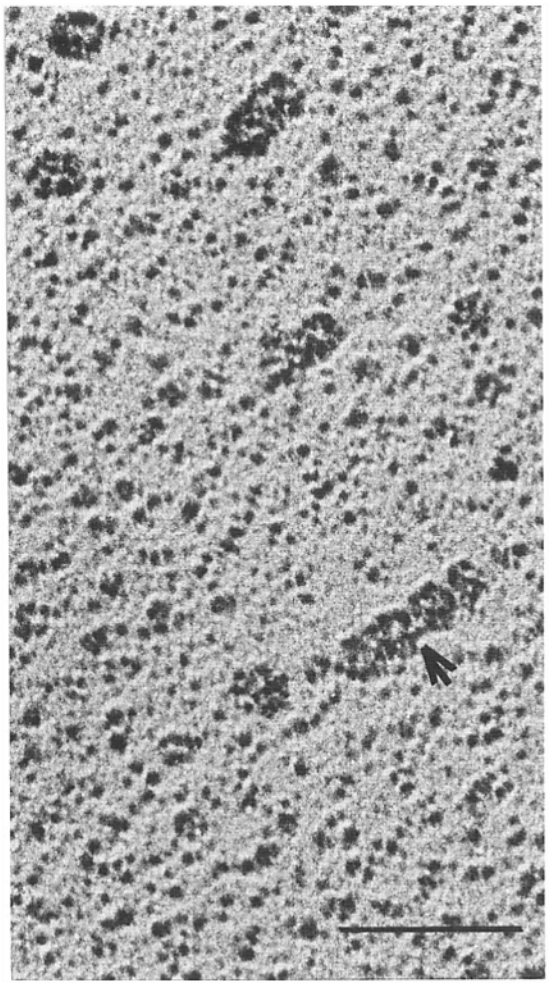


Figure 3. Electron microscopy of avidin polymers. Rotary-shadowed mixture of avidin polymers, including a tetramer (*arrow*) and 2 dimers of avidin. The dimensions of the rotary-shadowed image are larger than the actual molecular dimensions. Bar, 500 Å.

avidin monomers to the same end of another monomer or polymer. There were no obvious structural differences between avidin polymers that had been covalently cross-linked with BS^3 and those that were never cross-linked.

The micrograph shown in Fig. 4 *A* corresponds to BS^3–cross-linked material from an HPLC gel permeation fraction that appeared to be predominately dimers from the PAGE profile (*inset*, Fig. 4 *B*, fraction *5*). Several micrographs (including Fig. 4 *A*) were used to measure the polymer length distribution in this sample for comparison to the PAGE band distribution. If the length distribution is arbitrarily divided into the three major peaks, then 29% of the polymers lie in the area with the shortest length with an average of 49.4 Å corresponding to monomers. The middle peak includes 45.2% of the polymers and has an average length of 92.7 Å corresponding to dimers. The last peak includes 21.9% of the polymers and corresponds to trimers with an average length of 135.8 Å. The percentages of polymers calculated to fall in each of the three peaks compares very reasonably with the distribution of Coomassie stained proteins in the same HPLC fraction after PAGE (*inset*, Fig. 4 *B*; fraction *5*).

Binding of DNP-C_{12}-Biotin-BS^3-Avidin Monomer to FITC-IgE

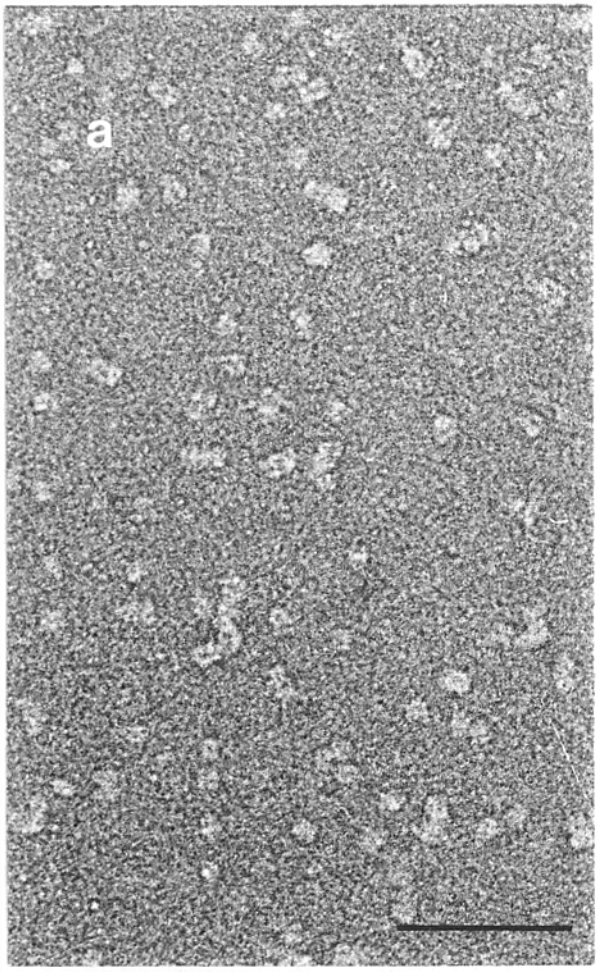
We showed previously that ~2 DNP-C_{12}-biotin conjugates bind per avidin monomer and that these bind at opposite faces of the nearly cubic protein (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). A similar stoichiometry was found for DNP-C_{12}-biotin bound to BS^3-cross-linked avidin monomers (see Materials and Methods). Equilibrium binding of this antigen to FITC-IgE in solution was measured with a fluorescence quenching method (Erickson et al., 1986). The data (not shown) indicate that binding and cross-linking occurs with similar intrinsic (K) and cross-linking (K_x) affinity constants, $K \sim K_x \sim 8 \times 10^6\ M^{-1}$. Similar results were obtained previously for the equilibrium binding of (DNP-C_{12}-biotin)-avidin monomer ($K \sim K_x \sim 10 \times 10^6\ M^{-1}$; Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). These data imply that the BS^3 modification does not affect the ability of (DNP-C_{12}- biotin)-avidin to bind bivalently to anti-DNP IgE in solution.

Degranulation Studies

The BS^3-cross-linked and fluorescamine-labeled avidin polymers were electroeluted from a nondenaturing polyacrylamide gel, and bands corresponding to six avidins or less could be isolated in sufficient quantities to test in degranulation assays. The electroeluted polymers were rerun in the same gel system used for the initial separation to assess their purity and stability (Fig. 5). Limited amounts of material prevented overloading these gels, but the electroeluted bands appear to be at least ~80% pure based on these results.

The electroeluted polymers were tested for their ability to stimulate degranulation of RBL cells sensitized with monoclonal anti-DNP IgE. As shown in Fig. 6, dimeric through hexameric avidin antigens produced dose-response curves that are superimposable within experimental error, while monomer stimulated a poorer response at all doses. In this experiment, only the monomer and dimer species were recovered in sufficiently high enough concentrations to observe a clear maximum in their dose-response curves, and these samples were not tested at the same lowest doses as the other polymers. In other experiments the polymer species were compared at the same doses, and the dose-response curves for dimers through hexamers were consistently superimposable in the dose range of 5×10^{-10} to 5×10^{-7} M DNP (data not shown). These general features were reproduced with different polymer preparations including preparations where there was sufficiently little fluorescamine that the DNP concentrations could be estimated directly from DNP absorbance (data not shown). BS^3 and fluorescamine-labeled polymers that had no DNP-C_{12}-biotin bound were purified in a parallel manner and did not stimulate degranulation when tested over a similar range of protein concentrations (data not shown). Within our experimental limitations, we conclude that the hexameric avidin antigen, of rigid length $\geq$240 Å, is very active in stimulating degranulation, as active as the dimeric avidin antigen of length ~80 Å.

BS^3-cross-linked, fluorescamine-labeled (DNP-C_{12}-biotin)-avidin monomers consistently triggered a poorer degranulation response than the polymers (see Fig. 6), and this was also much less than that seen with unmodified (DNP-



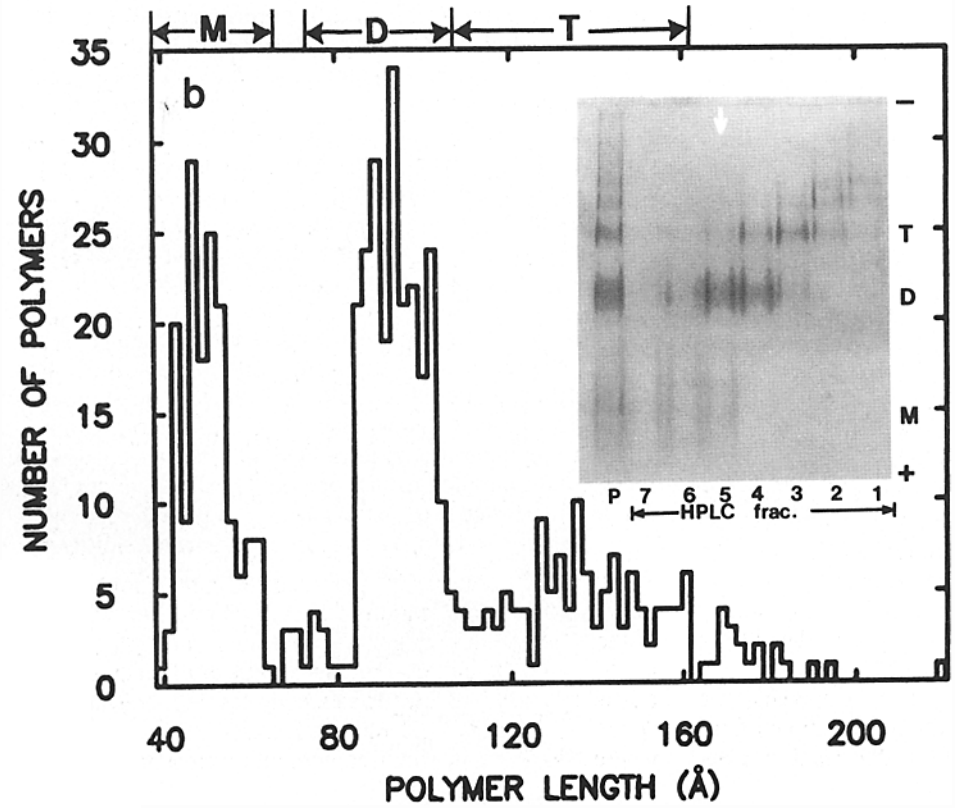


Figure 4. Correlation between the molecular mass and the length of avidin polymers. (*A*) Negatively stained preparation of a gel permeation HPLC fraction, which contained predominantly dimers, from a BS^3-avidin polymer mixture. (*B*) Distribution of polymer lengths determined from electron microscopy in a predominately dimer fraction from gel permeation HPLC (*inset*, *arrow*). The polymer lengths are plotted as described in Materials and Methods. The arrows indicate arbitrary divisions of the distribution into monomer (*M*), dimer (*D*), and trimer (*T*), as described in the text. (*Inset*) Anionic nondenaturing PAGE of an unfractionated polymer mixture (lane *P*) and fractions from a Superose 6 gel permeation HPLC column (lanes *1–7*). Bar, 500 Å.

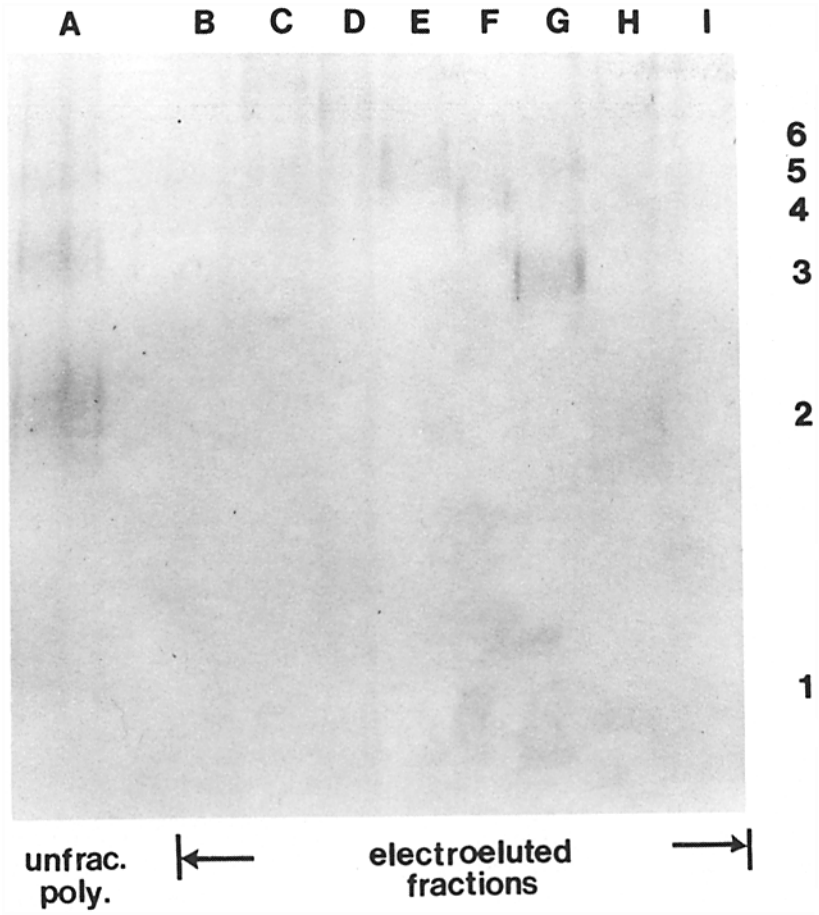


Figure 5. Nondenaturing PAGE of DNP-C_{12}-biotin-BS^3-avidin polymers purified by electrophoresis and electroelution. Lane *A* represents an unfractionated mixture of polymers. Lanes *B–I* correspond to protein bands that were excised from an anionic nondenaturing gel of a similar polymer mixture, electroeluted, and rerun in the same gel system. The numbers on the right indicate the location of monomer through hexamer. Less than 1 μg of protein was run in lanes *B–I*, and the gel was stained with Coomassie Blue.

C_{12}-biotin)-avidin monomers (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). The difference was not due to the fluorescamine modification since fluorescamine-labeled avidin monomer, which was not covalently crosslinked with BS^3, appeared to induce a good response, comparable to that of avidin dimer after electroelution (data not shown). However, both (DNP-C_{12}-biotin)-BS^3-avidin monomer that had been purified from a polymer preparation by gel permeation HPLC instead of electroelution and (DNP-C_{12}-biotin)-BS^3-avidin monomer that had never been reacted with bis-biotin gave a level of release similar to the electroeluted BS^3 monomer shown in Fig. 6. Therefore it appears that the BS^3-cross-linking step causes the avidin monomer to become a poorer stimulus.

We investigated the nature of the BS^3-induced change in the antigen potency by reacting avidin with a cleavable covalent cross-linker, DTSSP, which has a structure analogous to BS^3 but includes a disulfide bond that can be reduced after cross-linking. As shown in Fig. 7, we found that the (DNP-C_{12}-biotin)-DTSSP-avidin caused more release than the BS^3 analogue, and this was slightly enhanced after reduction with dithiothreitol (DTT). The same treatment did not significantly alter the level of degranulation triggered by (DNP-C_{12}-biotin)-BS^3-avidin nor that triggered by multivalent antigen, DNP_{23}-BSA (data not shown). Thus it appears that BS^3-mediated intramolecular cross-linking of monomeric avidin causes subtle structural alterations that result in a reduced ability to stimulate degranulation.

Redistribution and Mobility of Receptor-bound FITC-IgE Cross-linked by (DNP-C_{12}-Biotin)-BS^3-Avidin Polymers

The ability of haptenated avidin polymers to redistribute cell surface FITC-IgE into patches and induce internalization of these complexes was examined by fluorescence microscopy. At 4°C, concentrations of (DNP-C_{12}-biotin)-BS^3-avidin trimers and larger polymers that were optimal for triggering degranulation (10^{-7} M DNP) caused very little patching of FITC-IgE and no detectable endocytosis after 3 h incubation. At 25°C some patches of FITC-IgE were noted after 2 h, and fluorescence localizing to the interfaces of agglutinated pairs of cells was also evident at this time. More extensive patching was noted at longer incubation times. The (DNP-C_{12}-biotin)-BS^3-avidin monomers showed effects similar to the polymers at both 4 and 25°C. These observations are qualitatively similar to those made previously with uncross-linked (DNP-C_{12}-biotin)-avidin monomers and contrast with the more rapid patching seen with either multivalent antigens or tetravalent (DNP-Sac_2-biotin)-avidin (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). Little evidence for internalization of FITC-IgE was seen with any of these BS^3-avidin antigens, even after incubation at 37°C for >60 min. Quantitative measurements of FITC quenching caused by entry into low pH endocytic vesicles indicated that ≤10% of the FITC-IgE was internalized under conditions where >40% was internalized with multivalent antigen (data not shown).

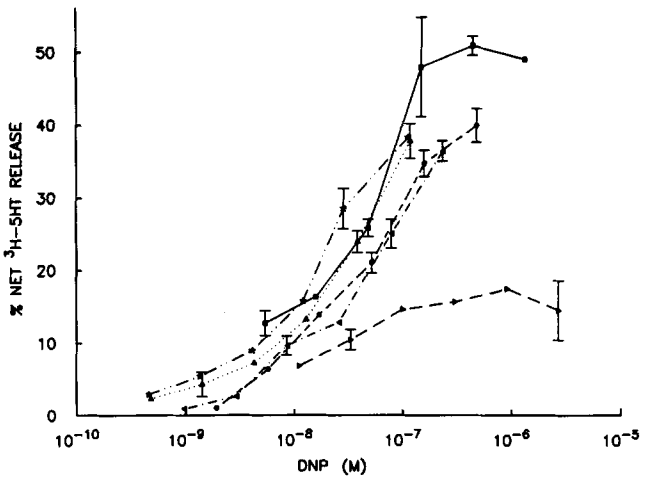


Figure 6. Net ^{3}H-5HT release stimulated by electroeluted BS^3-avidin monomer (▶), dimer (■), trimer (●), tetramer (◀), pentamer (★), and hexamer (▲) fractions. DNP concentrations were estimated from fluorescamine-avidin fluorescence as described in Materials and Methods. Spontaneous release of 6.0% was subtracted from the total release for each data point to obtain the net release. Each point is the average of duplicate measurements, and the bars represent the range of duplicates that were outside the range covered by the symbol.

We attempted to estimate what fraction of the cell surface IgE was being cross-linked by (DNP-C_{12}-biotin)-BS^3-avidin with the technique of fluorescence photobleaching recovery (Menon et al., 1986*a*, *b*). The ability of the monomer and polymer fractions to induce immobilization of receptor-bound FITC-IgE was measured, and the results are summarized in Table I. Addition of a concentration of avidin polymers that stimulated nearly maximal degranulation (10^{-7} M DNP) caused a reduction in the mobile fraction of FITC-IgE from ~60 to ~25%, while addition of a suboptimal dose of

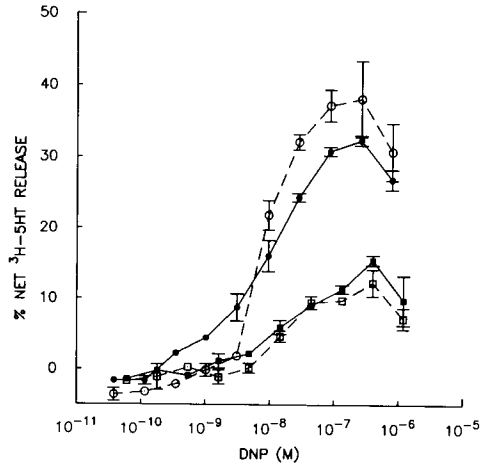


Figure 7. Degranulation stimulated by (DNP-C_{12}-biotin)-DTSSP-avidin monomer (○, ●) and (DNP-C_{12}-biotin)-BS^3-avidin monomer (□, ■) in the presence (○, □) and absence (●, ■) of 1 mM DTT. Both avidin samples were covalently cross-linked, haptenated with DNP-C_{12}-biotin, then subjected to nondenaturing PAGE (anionic system), and electroeluted. DNP concentrations were measured by absorbance. The dose-response curves are plotted as described in Fig. 6 after subtraction of 15% spontaneous release.

Table I. Percent Fluorescence Recovery Values from Fluorescence Photobleaching Recovery Measurements

Sample	Percent fluorescence recovery*
FITC-IgE	57.9 ± 4.0 (55)
FITC-IgE + 0.03 μM DNP-C_{12}-biotin-BS^3 polymers‡	67.8 ± 3.6 (17)
FITC-IgE + 0.1 μM DNP-C_{12}-biotin-BS^3 polymers	25.3 ± 3.7 (38)
FITC-IgE + 0.1 μM DNP-C_{12}-biotin-BS^3 monomer§	28.2 ± 5.1 (34)

* Percent fluorescence recovery values are expressed as the mean ± SEM (number of cells analyzed).
‡ This sample used was fractionated by gel permeation HPLC and, as shown by nondenaturing PAGE, contained only trimers and higher oligomers of avidin. The concentrations of DNP, obtained from absorbance, are expressed here. In a degranulation assay performed with the same HPLC fraction, a DNP concentration of 0.1 μM triggered a near-maximal response, and 0.03 μM DNP triggered a response that was ~30% of that response.
§ This sample was purified by gel permeation HPLC. 0.1 μM DNP stimulated nearly maximal degranulation for this antigen, but this maximum was considerably less than that obtained with the polymeric avidin antigen (similar to Fig. 6).

polymers resulted in no decrease in the mobile fraction. These results suggested that the polymer-stimulated degranulation was not due to a small population of cross-linked receptors (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). It is notable that a concentration of (DNP-C_{12}-biotin)-BS^3-avidin monomer (10^{-7} M DNP) that stimulated some degranulation, but consistently gave a poorer response than the polymers (Fig. 6), caused almost as much immobilization as the polymers (Table I). This immobilization is consistent with the ability of the (DNP-C_{12}-biotin)-BS^3-monomer to cross-link FITC-IgE in solution, as judged from binding experiments, and on the cell surface, as judged by fluorescence microscopy. It appears that the poorer degranulation response to the BS^3-monomer cannot be accounted for by an inability of this antigen to cross-link cell surface IgE, and that the magnitude of the degranulation response does not always correlate directly with the extent of cell surface cross-linking.

Discussion

These studies used avidin polymers as "molecular rulers" to determine how far apart cross-linked receptors for IgE can be held and still deliver a triggering signal. The formation of the polymers with bis-biotin-1,12-diaminodecane was based on the work of Green (1975), and we developed methods for preparing a series of monomers through hexamers, 40–240 Å in length, that were stable and could be isolated by preparative electrophoresis as outlined in Fig. 2. For our purposes, the avidin polymers were haptenated with DNP-C_{12}-biotin for use with a monoclonal IgE anti-DNP, but in principle the same strategy could be employed with a wide variety of biotinylated ligands. The essential features of the polymers for their use as molecular rulers are: (*a*) stability, (*b*) well-defined size and length, (*c*) linearity and rigidity, and (*d*) bivalency. These features are discussed in the following paragraphs.

The stability of the polymers at various stages in their

preparation can be assessed by nondenaturing PAGE and gel filtration. When gel filtration fractions of the unmodified polymers were run in PAGE (Figs. 1 and 4), they continued to give similar profiles that were consistent with their molecular weight estimated from gel filtration after several weeks of storage at 4°C. Fluorescamine labeling caused polymer breakdown (predominantly monomers and dimers after 2–3 wk), but this could be prevented by intramolecular cross-linking with BS^3. After this treatment, monomers–hexamers could be purified by preparative electrophoresis, and the isolated species rerun in PAGE showed no evidence of redistribution to smaller sizes (Fig. 5).

We were able to show that polymers separated by molecular mass with gel filtration or electrophoresis have the corresponding lengths expected for linear avidin polymers as visualized by electron microscopy (Fig. 4). We originally had some concern that possible heterogeneity of the charge on avidin due to different degrees of fluorescamine or BS^3 labeling could influence the electrophoretic separation. However, the appearance of the polymers as a banded rather than smeared pattern after PAGE suggested that there was no gross heterogeneity in the charge on the protein, and the consistency between the electrophoretic and gel filtration separations confirmed that the separation is predominantly by size. The average polymer lengths measured in electron micrographs for monomer, dimer, and trimer (49.4, 92.7, and 135.8 Å, respectively) are in reasonable agreement with those measured by Green (41 or 55, 82, and 123 Å; Green, 1971), considering the uncertainties in this measurement.

Our electron microscopy also agrees well with Green's characterization of the polymers as predominantly linear and rigid. The preparation of avidin polymers described here was designed to give a mixture of shorter polymers than those visualized by Green, and under these conditions we saw virtually no branched polymers. There also appeared to be relatively little curvature in the longer polymers of $n \leqslant 8$ visualized by us or by Green et al. (1971); the inclusion of a covalent cross-linker in our preparation probably minimizes polymer flexibility.

We have previously provided strong evidence that complexes of avidin with the DNP-C_{12}-biotin ligand act as bivalent antigens (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication), primarily because only about two DNP-C_{12}-biotin groups remain tightly associated with avidin after dialysis, and these appear to be at sites on opposite 55 × 55 Å faces of avidin that are separated by the 41 Å dimension. Previous electron microscopy studies showed that only one Fab fragment of polyclonal anti-DNP antibodies could bind at each face of (DNP-C_{12}-biotin)-avidin in ≥97% of these avidin molecules (Green, 1975). In those studies dialysis was not used to remove more weakly bound DNP-C_{12}-biotin molecules, so in that case it appears that steric repulsion prevents more than one Fab from occupying each biotin-binding face. Our fluorescence microscopy studies indicated that cross-linking by the (DNP-C_{12}-biotin)-avidin antigens causes a much slower redistribution of IgE receptors into visible patches than that seen with multivalent antigens (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). The same arguments apply here for the bivalency of DNP-C_{12}-biotin bound to the avidin-capped ends of avidin polymers. The degranulation results presented in this study help eliminate the possibility that binding of two Fab arms at one face of the avidin is responsible for stimulating degranulation, since this type of binding should be equally available to the BS^3-avidin monomer and polymers, and the BS^3-monomer is a much poorer triggering reagent.

One problem that is introduced with polymer formation is the possibility of introducing DNP-C_{12}-biotin internal to the polymer chain if the internal biotin binding sites are incompletely occupied by bis-biotin. Several lines of evidence argue against this type of multivalent polymer making a significant contribution in the cellular response to the avidin polymers. First, addition of hydroxyazobenzoic acid (Green, 1965) after the initial bis-biotin binding to avidin indicated that virtually all (97%) of the biotin-binding sites were filled at this step (data not shown). Second, the results of Kane et al. (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication), indicating that DNP-C_{12}-biotin cannot bind tightly to a biotin binding site adjacent to another bound DNP-C_{12}-biotin, suggest that it is difficult for a DNP-C_{12}-biotin molecule to bind adjacent to a bis-biotin molecule of similar length in a single-linked polymer. Finally, the superimposible dose-response curves for degranulation stimulated by dimers through hexamers of avidin purified by electroelution (Fig. 6 and unpublished results) indicate that the triggering activity seen with the largest polymers is not due to contamination of these samples with small amounts of the shorter polymers.

The arguments described above suggest that there are not a large number of DNP-C_{12}-biotin molecules bound in the middle of the avidin polymers, but cannot eliminate the possibility that a small number of "multivalent" polymers are responsible for much of the degranulation response in all of the electroeluted samples, since a small number of appropriately cross-linked receptors can stimulate maximal degranulation in these cells (Fewtrell, 1985). We think that this explanation of our results is unlikely, since it would require that the same amount of this minor activating species is present in each polymer fraction to give rise to the superimposable dose-response curves. We attempted to assess the fraction of active species by carrying out fluorescence photobleaching recovery measurements on cells saturated with FITC-IgE and treated with concentrations of the (DNP-C_{12}-biotin)-BS^3-avidin polymer mixture which give maximal degranulation. Cross-linking by the polymer mixture results in a large decrease in the mobile fraction of IgE (Table I) that is similar to that seen previously with (DNP-C_{12}-biotin)-avidin (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication). This reduction in mobility indicates that a large proportion of the receptors are cross-linked and immobilized at DNP concentrations which give optimal degranulation. Since immobilization has been shown previously to reflect the interaction of the IgE–receptor complex with cellular component(s) that accompanies early events in signal transduction (Menon et al., 1986*a*, *b*), it is likely that a large fraction of polymers are capable of cross-linking IgE–receptor complexes in a manner that can trigger cellular degranulation.

The (DNP-C_{12}-biotin)-BS^3-avidin monomer stimulates poorer degranulation than do dimers through hexamers or unmodified (DNP-C_{12}-biotin)-avidin monomers, and this is caused by the BS^3 cross-linking step. The poorer response of the cells to BS^3-avidin monomer does not appear to be

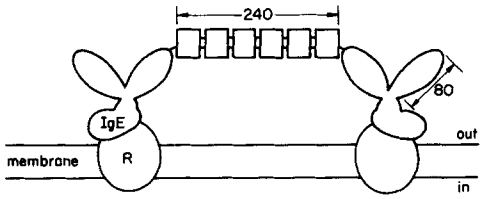


Figure 8. Schematic diagram showing a hypothetical model for hexameric avidin cross-linking two IgE-receptor complexes. Distances shown are in Å. *R* represents the receptor for IgE. Sources of size estimates are indicated in the text.

due to an inability of the antigen to bind and cross-link IgE based on the solution binding data, the significant immobilization of receptor-bound FITC-IgE (Table I), and the patching of IgE-receptor complexes observed by fluorescence microscopy. The BS^3 monomer may be near a threshold capability to stimulate cellular responsiveness, and thus may behave somewhat like the peptide bivalent antigens previously characterized (Kane et al., 1986), some of which also cross-link efficiently on the cell surface (Erickson et al., 1986). The apparent lack of correlation between the magnitude of immobilization and that of degranulation is somewhat surprising in light of previous results (Menon et al., 1986*a*, *b*), and so it may be that the effects of cross-linking by the monomeric avidin antigen differ from those of the higher polymers. For example, there is now evidence that RBL cells can be desensitized (Seagrave et al., 1987 and our unpublished results), and the monomeric avidin antigen may stimulate an inactivation signal that competes effectively with the activation signal. Alternatively, shorter bivalent ligands may be very inefficient at triggering cellular degranulation because they generally hold IgE-receptor complexes too close together. The BS^3-avidin monomer may approach this threshold because the chemical cross-links have constrained the internal flexibility of avidin.

Measurement of Interreceptor Distances from Avidin Polymer Length

When the avidin polymers are bound bivalently to receptor-bound anti-DNP IgE, the polymer length should provide a measure of the distance between the combining sites of the antibodies it cross-links. While this is not a direct measure of interreceptor distances, the following information on IgE-receptor complexes permits us to propose a model relating polymer length and receptor-receptor separation as illustrated in Fig. 8. (*a*) The antibody combining sites are located at the ends of the Fab arms of the Y-shaped IgE molecule, ~80 Å from the NH_2-terminal end of the hinge domain, $C_\varepsilon 2$ (Dorrington and Bennich, 1978). (*b*) Steady-state fluorescence anisotropy measurements indicate that the Fab arms of the IgE in solution have limited segmental flexibility, and that some modes of motion are retained when the IgE is bound to its receptor (Slattery et al., 1985). More recent studies using nanosecond fluorescence depolarization measurements, which provide more detailed information, indicate that the segmental motion of receptor-bound IgE is very limited (Holowka, D., T. Wensel, B. Baird, and L. Stryer, manuscript in preparation). (*c*) Fluorescence resonance energy transfer measurements indicate that IgE binds to its receptor in a "bent" conformation, with the IgE interacting with its receptor somewhere near the junction of the $C_\varepsilon 2$ and $C_\varepsilon 3$ domains (Perez-Montfort and Metzger, 1982; Holowka et al., 1985) and with Fab arms projecting upward from the cell surface (Baird and Holowka, 1985). These data are consistent with the nanosecond depolarization results and indicate that the Fab arms are not free to undergo extensive "wagging" motions that could bring the antibody combining sites closer to the plasma membrane. (*d*) Resonance energy transfer measurements between membrane-bound IgE-receptor complexes cross-linked by the monoclonal anti-IgE A2 yielded minimal distances of 92–102 Å between cross-linked IgE molecules (Menon, A. K., D. Holowka, and B. Baird, manuscript in preparation), suggesting an upper limit for the receptor radius of 45–50 Å. Electron micrographs of purified IgE-receptor complexes appear to be consistent with this result (Kinet, J-P., M. Phillips, V. Schumaker, and H. Metzger, personal communication). In general, resonance energy measurements of average distances between IgE-receptor complexes cross-linked by flexible monoclonal or polyclonal anti-IgE antibodies showed little evidence for stable interactions between directly bridged receptors (Menon, A. K., D. Holowka, and B. Baird, manuscript in preparation).

Although there are other ways to portray the polymer cross-linked IgE-receptor complexes than the model of Fig. 8, many of these other structures are less consistent with results described here. For example, these complexes can be drawn with the IgE molecules rotated $\leqslant 180°$ around an axis that is at the junction between IgE and avidin and is perpendicular to the plane of the membrane, such that the empty IgE combining sites are adjacent. This structure might barely allow contact between receptors cross-linked by an avidin hexamer if the Fab arms involved in antigen binding project directly away from each other, but this model also introduces new steric strain in the binding of the avidin-linked DNP hapten to IgE: the intrinsic affinity of anti-DNP IgE for these antigens as measured in solution ($K \cong 10^7\ M^{-1}$) is at least an order of magnitude lower than that for the same DNP-C_{12}-biotin ligand when it is not bound to avidin (Kane, P., D. Holowka, and B. Baird, manuscript submitted for publication), indicating that the biotin binding site on the end face of avidin restricts the accessibility of the DNP moiety and thus limits the amount of rotation that can occur at this junction. Even if such compact complexes could exist in stable form, it is likely that steric constraints would prevent them from participating in the formation of larger chains of IgE-receptor complexes, and dimeric complexes are expected to cause poor triggering (Fewtrell and Metzger, 1980) and no immobilization (Menon et al., 1986*b*).

From these considerations, it seems unlikely that a polymeric avidin antigen $\geqslant 200$ Å in length should permit the receptors it cross-links to make stable contact. This conclusion is important, since avidin pentamers and hexamers, which are $\geqslant 200$ and 240 Å in length, respectively, are as active in stimulating degranulation as shorter avidin antigens. However, it is difficult to completely rule out the possibility that transient interactions between even hexamer cross-linked receptors might occur because of the uncertainties inherent in structural information derived from spectroscopic measurements on a dynamic system. Other techniques capable of mapping receptor-receptor spacings, such as resonance energy transfer (Baird and Holowka, 1988) and backscat-

tered electron imaging of gold-labeled IgE in conjunction with scanning electron microscopy (Stump et al., 1988) should allow us to continue to search for possible interactions between avidin cross-linked receptors.

Implications for the Mechanism of Signal Transduction Leading to Degranulation

Metzger (1983) has suggested two broad classes of mechanisms by which receptor aggregation could initiate signal transduction leading to degranulation. He describes these two classes as "intrinsic mechanisms", which require aggregated receptors to make van der Waals contact to form an active site or produce a mutual conformational change, and "extrinsic mechanisms" which simply require receptors to be at a sufficiently high local concentration to facilitate interactions with other signal-generating molecules. It is difficult to distinguish between these different classes of mechanisms experimentally. Attempts to identify changes in the structure of the receptor after cross-linking (Perez-Montfort et al., 1983) or to reconstitute functionally effective receptors into liposomes (Metzger et al., 1984), two approaches to uncovering an intrinsic mechanism for receptor activation, have not yet provided unequivocal answers. Experiments aimed at capturing transient new molecular interactions after receptor aggregation with covalent cross-linking reagents have failed to show any evidence for receptor–receptor interactions (Holowka et al., 1980 and unpublished results). Our approach to distinguishing between extrinsic and intrinsic signal transduction mechanisms has used rigid spacers to gauge the interreceptor distances that permit stimulation of RBL cells. Our observations indicate that signal transduction does not require an intrinsic mechanism, at least in its simplest form where the directly cross-linked receptors must come into stable contact. Signal transduction via an intrinsic mechanism is still possible if receptors that are not directly cross-linked to each other are brought together. In one possible model, receptors from two different linear chains of cross-linked receptors could be brought into contact as a part of the cellular process of microclustering which can be initiated by very small IgE–receptor clusters (e.g., by $\geq$3 receptors aggregated by covalently cross-linked oligomers of IgE; Menon et al., 1984, 1986*a*). A second possible intrinsic mechanism would require contact between receptors that are at the ends of a single antigen-linked linear chain of three or more IgE–receptor complexes. The sole evidence in favor of this "sticky ends" model is the small increase in energy transfer seen in both RBL cells and plasma membrane preparations, when IgE–receptor complexes are cross-linked into chains longer than dimers with the monoclonal anti-IgE, A2 (Menon, A. K., D. Holowka, and B. Baird, manuscript in preparation).

Our results are completely consistent with an extrinsic mechanism of signal transduction, in which receptors never need to make van der Waals contact. Metzger (1983) envisioned this type of mechanism as operating via an increased local concentration of receptors which potentially could allow stable multivalent binding to a protein that binds very weakly to single IgE receptors. This mechanism is similar to that proposed for the activation of complement by the binding of Clq to cell surface antigen–IgG complexes (Porter and Reid, 1979). While this type of mechanism is possible for the polymer lengths we have tested, the cellular response to the longest polymers prompts us to propose a more general extrinsic mechanism. Since a single signal-transducing protein analogous to Clq would have to be very large to bind multivalently to IgE–receptor complexes separated by antigens 200–240 Å, we suggest that external receptor bridging causes multivalent stabilization of weak interactions with components that are effectively multivalent by virtue of being anchored to the cytoskeleton. A variety of studies have suggested an increased interaction between the cytoskeleton and IgE receptors after aggregation (Menon et al., 1984, 1986*a*, *b*; Robertson et al., 1986), and the immobilization of IgE receptors after cross-linking with avidin polymers indicates that these antigens also promotes such an attachment. It is not clear that interactions with the cytoskeleton are critical for signal transduction leading to degranulation, although previous experimental evidence is consistent with this possibility (Menon et al., 1986*a*, *b*; Robertson et al., 1986). Other evidence that bridging IgE–receptor complexes to each other may provide a means by which the receptors are induced to interact with other cell components comes from studies by Voisin and colleagues which indicate that tethering of Fc receptor complexes to histocompatibility antigens on mast cells is sufficient to trigger cellular degranulation (Daeron et al., 1975).

A second type of extrinsic mechanism modeled by DeLisi (1981) portrays cross-linking as a means of forcing two receptors to remain within an "active radius" for a sufficiently long time (longer than the time allowed by encounters of single freely diffusing receptors) to transmit a signal. A strict application of this model to our system would require a very long active radius for signal transduction, but presumably less than the average interreceptor distance calculated to be only $\sim$500 Å on these cells (Metzger, 1983). A similar model may apply if cross-linking receptors can significantly alter their rotational diffusion, perhaps stabilizing them in a favorable orientation for a time sufficient for signal transduction. Although some initial work on the rotational dynamics of the IgE receptor has been carried out (Zidovetzki et al., 1986), little is known about the importance of changes in rotational dynamics after cross-linking for signal transduction.

In summary, we have shown that cross-linking of IgE–receptor complexes with antigens that probably do not permit stable contact between directly bridged receptors provides a good stimulus for triggering cellular degranulation. The use of polymeric avidin antigens to maintain long interreceptor distances during stimulation has made some types of intrinsic mechanisms for signal transduction unlikely, but several other types of mechanisms are possible. Future studies will be aimed at distinguishing these.

We are grateful to Dr. Richard Henderson for suggesting the use of polymeric avidin as rigid spacers for this study and to Dr. N. Michael Green for generously providing helpful advice and several of the biotin derivatives. We appreciate the assistance of Dr. John Telford who performed the electron microscopy.

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