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IMMUNOGENICITY OF POLYMERS OF H ANTIGEN OBTAINED BY DIFFERENT METHODS

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The properties of two polymers obtained from flagellin by different methods — precipitation by ammonium sulfate followed by centrifugation or treatment with glutaraldehyde in solution followed by gel chromatography — were studied. Molecules of the former (POL) and the latter (GLUT) are similar in shape but POL has a higher molecular weight. The preparations contain a common H antigen and are similar in serologic activity. POL has very high "priming" activity for mice. GLUT is highly immunogenic only within a narrow dose range, is less immunogenic than POL, and differs from POL in forming a certain quantity of 7S antibodies.

KEY WORDS: *flagellin; immunogenicity; polymer antigen.*

One of the basic principles of modern immunology is that high-polymer molecules are much more immunogenic than low-polymer or monomer molecules. The concept of high polymerism is not completely quantitative: the relations between the properties of different polymers of the same protein and to what extent they depend on the method of preparation of the polymer are not yet known. Comparison of different polymers is essential to fill in the gaps of our knowledge of concepts such as "high immunogenicity," "T independence," and "tolerogenicity," used in connection with the degree of polymerization of antigens.

The object of this investigation was to compare polymers of flagellin obtained by two different methods.

EXPERIMENTAL METHODS

Three preparations of flagellin, namely flagellae (FLA), flagellin monomer (MON), and the spontaneously formed polymer (POL), were prepared by the methods described previously [1, 4]. The methods consisted of separating flagellae from bacterial cells of *Salmonella typhi* strain T-55-01 by intensive shaking of the culture and centrifugation, dissolving the flagellae at pH 3.5 (with the formation of MON), and treatment of the MON solution with 15% $(NH_4)_2SO_4$ (to precipitate POL). The glutaraldehyde polymer of flagellin (GLUT) was prepared by incubating MON in a concentration of 10 mg/ml in 0.1 M phosphate buffer, pH 6.8, with 0.025% glutaraldehyde for 30 min at 20°C [6]. The polymer was separated from unpolymerized protein on a column with Sephadex G-50. The preparations were purified by chromatography on a column with Sepharose 2B.

By marking the column with Newcastle desease virus (mol. wt. 7×10^7), mouse encephalomyocarditis virus (mol. wt. 10^7), dextran preparations with mol. wt. of 2×10^6, 5×10^5, and 4×10^4, and with deoxyribose with mol. wt. 134 daltons the molecular weight of the flagellin preparations could be determined.

The serologic specificity of the preparations thus obtained was determined by radial immunodiffusion tests with rabbit serum against flagellin. The serologic activity of the prep-

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arations was determined by a radioimmune method with [^{125}I]flagellin.

The immunogenicity of the flagellin was determined by immunizing mice with two intravenous injections at an interval of 4 days; blood for determining the antibody titer was taken 96 h after the second injection of the flagellin. Titration was by the passive hemagglutination method.

EXPERIMENTAL RESULTS

The molecular weight of POL was (19-20) × 10^6, of FLA (15-16) × 10^6, GLUT 13 × 10^6, and MON 4 × 10^4 daltons. Electron microscopy showed that the FLA molecules are shaped like long twisted threads, whereas the POL and GLUT molecule were straighter and thicker rod-shaped segments (Fig. 1). The similarity between the POL and GLUT molecules shows that polymerization followed a similar course in the two cases; presumably the role of glutaraldehyde was to stabilize the large molecules formed spontaneously.

All the preparations had a common and powerful H antigen, as was shown by the immunodiffusion test; there was no difference in serologic specificity. The serologic activity of the preparations was clearly detectable in concentrations inhibiting attachment of the labeled flagellin to antibodies by 50% (IC_{50}). For MON, IC_{50} was 0.039 μg/ml, for POL 0.031 μg/ml, and for GLUT 0.06 μg/ml. The difference between the values of IC_{50} were not statistically significant.

Comparison of the immunizing properties of the preparations revealed differences between POL and GLUT. The immunogenicity of the preparations increased with an increase in their molecular weight (Fig. 2): POL was more immunogenic than GLUT.

POL possessed strong "priming" activity in a dose as low as 0.1 μg per mouse. The intensity of the immune response to POL was determined by its dose at the "reacting" injection (Table 1). GLUT began to exhibit its "priming" action in a dose of 6.0 μg. The intensity of antinody synthesis increase in both the 1st and the 2nd dose of GLUT (this rule also was characteristic of the action of MON; see Table 1).

Dose-effect curves. For the two polymers (POL and GLUT), these differed sharply. The immunizing action of POL increased appreciably with an increase in the dose from 0.01 to 0.1 μg; with a further increase in the dose titer remained at about the same level. GLUT in a dose of 1 μg immunized almost as weakly as MON, in a dose of 10 μg almost as strongly as POL, and in a dose of 60 μg it induced immunodepression (Fig. 2).

The polymers also differed in their ability to induce the formation of 7S antibodies, detectable by their resistance to the action of 2-mercaptoethanol. POL induced the formation

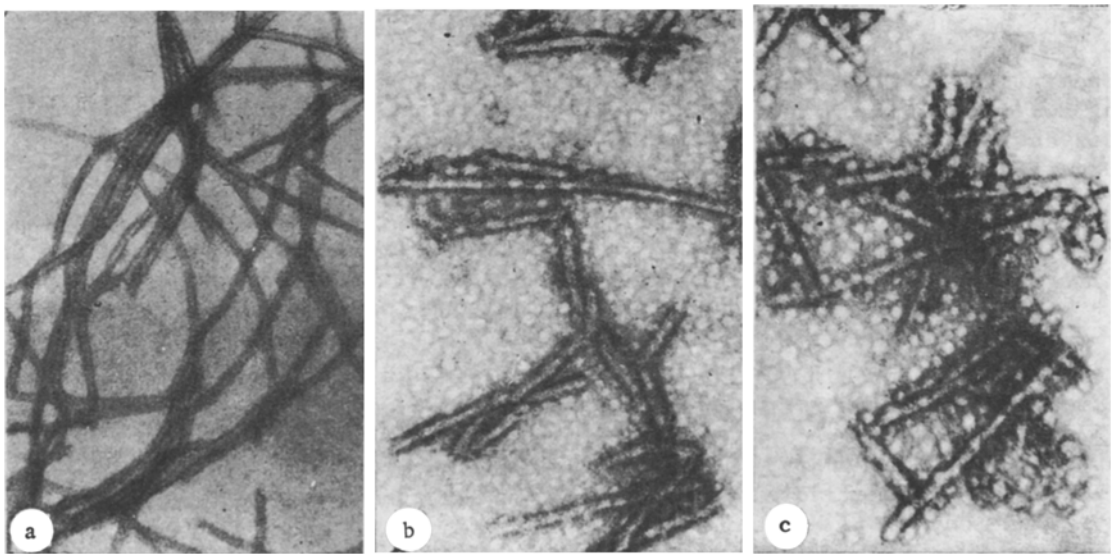

Fig. 1. Electron microscopy of flagellin preparations: a) FLA; b) POL; c) GLUT; 60,000×.

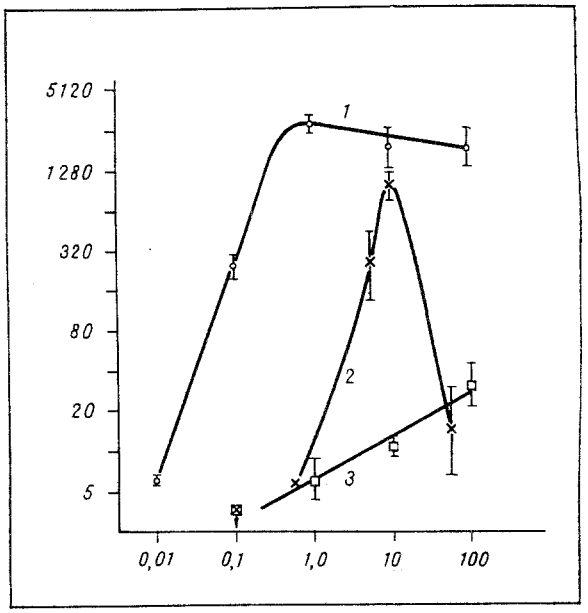

Fig. 2. Antibody titers in mice as functions of dose of flagellin preparations: 1) immunization with POL; 2) immunization with GLUT; 3) immunization with MON. Results of experiments in which "priming" and "reacting" doses of preparations were equal. Abscissa, reacting dose of test preparations (in μg); ordinate, reciprocals of antibody titers in passive hemagglutination test (micromodification).

TABLE 1. Role of "Priming" and "Reacting" Doses of Flagellin during Immunization with Different Preparations

Antigen	Scheme of immunization			
	equal doses at 1st and 2nd injections		different doses at 1st and 2nd injections	
	dose, μg per mouse	mean titer	dose, μg per mouse	mean titer
POL	0,01±0,01	1:6.5 (1:6.0—1:6,6)	0,1±0,01	1:6.6 (1:5,1—1:8,5)
	0,1—0,1	1:240 (1:200—1:288)	0,1—0,1	1:237 (1:185—1:304)
	1.0—1,0	1:2630 (1:2190—1:3160)	0,1—1.0	1:1783 (1:1380—1:2188)
	10+10	1:1780 (1:1280—1:2570)	0,1+10	1:3311 (1:2344—1:4677)
	100+100	1:1778 (1:1300—1:2500)	0,1+100	1:2188 (1:1749—1:3236)
GLUT	0,6+0,6	1:5 —	0,1+0,6	1:5 —
	6,0+6,0	1:200 (1:100—1:398)	0,1+1,0	1:7,8 (1:4,2—1:14,4)
	10+10	1:794 (1:631—1:997)	0,1+10	1:4,9 (1:3,8—1:6,3)
	60+60	1:14.1 (1:6,2—1:32,3)	0,1+100	1:3,5 (1:3,5—1:6,7)
MON	1,0+1,0	1:6,2 (1:4,7—1:8,1)	10+1,0	1:34,6 (1:29—1:42)
	10+10	1:23,4 (1:19—1:29)	10+10	1:23,4 (1:19—1:29)
	100+100	1:34 (1:24—1:48)	10+100	1:35 (1:24—1:50)

Note. 1. 0.01 + 0.01 means that first dose was 0.01 μg per mouse and second "reacting" dose was 0.01 μg per mouse, and so on.
2. Titer shown in geometric mean values; confidence limits at P = 0.05 shown in parentheses.

of 19S antibodies virtually exclusively. GLUT, under optimal immunization conditions, induced formations of 7S antibodies in a titer of 1:128 (1:90-1:186) out of a total antibody titer of 1:3150 (1:2590-1:3950). It is interesting to note that ability to induce the formation of 7S antibodies is a characteristic feature of MON [7].

The two high-molecular-weight polymers (POL and GLUT) thus differed considerably from each other.

The differences in the level of immunogenicity can be explained by the differences in the size of the molecules. Usually the high immunogenicity of POL is explained on the grounds that its molecule, with its numerous determinants of the same kind, has high affinity for the surface of immunocompetent cells carrying receptors for the antigen [2]. There are fewer determinants on the smaller GLUT molecule; consequently, the affinity of GLUT for the immunocompetent cells is lower than the affinity of POL.

This difference was manifested particularly clearly after the first, "priming" injection of the preparations, i.e., after contact with the precursors of antibody synthesizing cells

(since flagellin polymer is a T independent antigen [3], it must be assumed that these are B lymphocytes). Differences in the number of specific determinants on the molecule of the polymeric antigen evidently are brought to light first on contact between the substance and the precursor cell carrying fewer specific receptors than the "primed" lymphocyte, differentiated for antibody formation.

The appearance of immunodepressive properties in GLUT not found under the same conditions in POL depends mainly on the glutaraldehyde residues remaining on the GLUT molecule. Changes in the molecule caused by these residues are evidently not great enough to disturb its serologic properties, but are sufficient to change interacton between GLUT and the immunocompetent cells. This suggestion arises by analogy with the results of an investigation of intensively acetylated flagellin, which lost its ability to cause antibody formation; its ability to induce hypersensitivity of delayed type, however, was intensified [5].

A general result of this investigation is to prove that polymers with different immunologic properties can be obtained from the same protein. Evidently the concept of "polymeric antigen" in every case requires more accurate specification of the properties of the concrete preparations. The further study of polymerization and aggregation of antigen molecules is essential so that the special features which make the molecule "highly immunogenic," "tolerogenic," and "T independent" can be characterized quantitatively, so that more accurately oriented changes can be brought about in the properties of antigen.

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ROLE OF DISTANT INTERACTIONS OF LYMPHOCYTES IN THE DEVELOPMENT OF ANTIBODY FORMATION *in vitro*

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To elucidate the nature of local intercellular interactions inhibiting the proliferation of antibody-forming cells (AFC) in culture, described previously, the possibility of realization of this effect at a distance was studied. A population containing many cells was shown to be capable of inhibiting, by its action *in vitro* at a distance, an increase in the number of AFC in a cell population separated from it by Millipore membranes impermeable to cells. This effect is also transmitted through a polymethylmethacrylate film, 5-10 μ thick, which does not allow the passage of proteins with a molecular weight of 150,000 daltons ([^{125}I]IgG antibodies) and certain ions ($^{51}CrO_4$), but is permeable to other low-molecular-weight substances.

KEY WORDS: *Antibody formation; distant interaction; proliferation.*

Many investigations have been carried out to study contact inhibition of cell proliferation in a monolayer, for this deals with one of the most important mechanisms of regulation of cell division in higher animals [6, 10]. More recently similar processes have been studied in suspension cultures also [7, 11]. In particular, in the writer's laboratory the

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