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In Vitro Comparison of the Biologic Activities of Monoclonal Monomeric IgA, Polymeric IgA, and Secretory IgA[1]

Kathryn B. Renegar,[2]*† Graham D. F. Jackson,[3]‡ and Jiri Mestecky*

Secretory IgA (S-IgA), a major humoral mediator of mucosal immunity, is a polymeric Ig containing an unusual extra polypeptide, secretory component (SC), added during transcytosis through epithelial cells. Polymeric S-IgA is more effective than monomeric IgA (mIgA) and IgG in neutralizing viruses. It is not known whether this increased efficacy is due solely to the polymeric structure of the molecule or whether SC itself makes S-IgA more efficient; a quantitative in vitro comparison of the biologic activities of S-IgA and pIgA has not been reported. We prepared purified pIgA and mIgA mAbs directed toward the H1 hemagglutinin of PR8 influenza virus and purified monoclonal S-IgA (made from monoclonal pIgA injected into a Lewis rat and collected as S-IgA from bile) and compared their abilities to carry out hemagglutination inhibition (HI) and neutralization of the infectivity of PR8 influenza virus in vitro. The polymeric Igs (pIgA and S-IgA) were 5 times more effective than mIgA in HI and 7 to 10 times more effective than mIgA in virus neutralization. Addition of SC to pIgA did not modify its ability to mediate HI and had only a minimal effect (S-IgA was 1.4 times more effective) on its ability to neutralize influenza virus in vitro. Trypsin preincubation partially abolished mIgA- or pIgA-mediated, but not S-IgA-mediated, viral neutralization. Thus, although S-IgA is more stable functionally than pIgA, the addition of SC does not influence, positively or negatively, the biologic activity associated with the Fab of S-IgA. ***The Journal of Immunology*****, 1998, 160: 1219–1223.**

IgA, the major contributor to humoral mucosal immunity, occurs in secretions as a dimer-bearing secretory component (SC)[4] (1). Secretory IgA (S-IgA) can effectively neutralize viruses (2–5) or toxins (reviewed in Ref. 6) at mucosal surfaces and can display antibacterial activity (7). The S-IgA molecule is more stable toward chemical and enzymatic cleavages than polymeric molecules not bearing SC (8, 9). It has been suggested that SC, in addition to adding stability, may enhance the neutralizing ability of the S-IgA molecule (10).

The dimeric form of the Ab molecule may also contribute to the effectiveness of S-IgA in mucosal protection. Polymeric IgA (pIgA) is 5 to 10 times more efficient than IgG in in vivo viral neutralization (11–13).[5] The use of IgG as the representative monomeric Ig in these studies, however, allows for the intrusion of isotype differences. A direct comparison of monomeric IgA (mIgA), pIgA, and S-IgA directed toward the same Ag is needed to determine the relative efficiencies of mIgA, pIgA, and S-IgA in viral neutralization.

In the studies reported in this paper we used purified m- or pIgA anti-influenza mAbs to determine the relative viral hemagglutination inhibition (HI) and virus-neutralizing abilities of mIgA vs pIgA. In addition, purified monoclonal S-IgA and purified monoclonal pIgA were compared to determine whether SC enhances the ability of IgA to neutralize influenza virus in vitro.

Materials and Methods

Animals

Retired breeder female BALB/c mice and male Lewis rats were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA), and maintained in an American Association for the Accreditation of Laboratory Animal Care-approved animal facility. Food and water were supplied ad libitum. Animal use protocols were approved by an institutional animal care and use committee.

Monomeric and polymeric IgA

Hybridomas producing monoclonal pIgA (H37-66-1) or mIgA (H37-50) directed toward the H1 hemagglutinin of PR8 influenza virus were gifts from Dr. Walter Gerhard of the Wistar Institute (Philadelphia, PA) and were generated as previously described (14). The hybridomas were propagated as ascitic tumors in mice. Monomeric and polymeric fractions of IgA were separated on an 80- × 3.5-cm Ultrogel AcA 22 (LKB, Rockville, MD) column and stored at 4°C. The presence of IgA in the fractions was determined by ELISA. The IgA content of the purified monomeric (150 μg/ml) or polymeric (400 μg/ml) pools was determined using an RID kit according to label instructions (ICN ImmunoBiologics, Lisle, IL). Hybridoma ZF11-15 (a gift from Dr. Zina Moldoveanu, University of Alabama-Birmingham), an IgA hybridoma that does not bind influenza virus, was propagated in mice and used as a control for the studies reported here.

Secretory IgA

Since pIgA acquires SC during hepatobiliary transport (1), mouse influenza-specific pIgA was passed through rat liver in vivo to become S-IgA. Mouse ascitic fluid containing mouse anti-influenza monoclonal pIgA (8.6 mg/ml) was centrifuged to preclude embolus formation, and 2 ml was injected into the tail vein of a Lewis rat anesthetized with ketamine and xylazine (87 mg of ketamine and 13 mg of xylazine per kg). The rat was maintained under deep anesthesia while the bile duct was cannulated, and bile was collected in 30-min fractions over a 3-h period. This procedure

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[4] Abbreviations used in this paper: SC, secretory component; S-IgA, secretory IgA; pIgA, polymeric IgA; mIgA, monomeric IgA; HI, hemagglutination inhibition; EBU, enzyme-linked immunosorbent assay binding unit; $TCID_{50}$, 50% infectious dose in tissue culture; HA, hemagglutination; CRBC, chicken red blood cell; HAU, hemagglutinating unit.

[5] K. B. Renegar and P. A. Small, Jr. Contribution of serum IgG to murine nasopharyngeal anti-influenza immunity. *Submitted for publication.*

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yielded 2 ml of bile. Bile salts were removed by treatment with Amberlite XAD-2 resin (15), and treated bile was stored in plastic tubes at 4°C. Bile was fractionated on the AcA 22 column, and the IgA content was determined by ELISA. Rat bile not containing mouse IgA was fractionated for use as a control in further studies. S-IgA-containing fractions were pooled and stored in glass at 4°C. The S-IgA pool was too dilute for Ab to be quantitated by radial immunodiffusion, so the amount of influenza-specific S-IgA present (5 μg/ml) was determined from parallel ELISA assays of the S-IgA and pIgA pools. The S-IgA pool was checked by ELISA immediately before each neutralization assay to monitor Ab activity.

ELISAs

A modification of the ELISA described by Renegar and Small (3) was used. PR8 (H1N1) influenza vaccine (a gift from Parke-Davis Co., Rochester MN) in PBS at a 1/50 dilution was dried onto 96-well flat-bottom microtiter trays (Linbro/Titer-Tek E.I.A. Microtitration plate, Flow Laboratories, Inc., Rockville, MD). Trays were blocked for 1 h with PBS/Tween containing 1% BSA (BSA for ELISA diluent applications was obtained from Sigma Chemical Co., St. Louis, MO). IgA-containing samples were incubated in wells for 90 min, the plates were washed, and the second and third Abs were added and incubated in a similar manner. Affinity-purified goat anti-mouse IgA (Sigma) was the secondary Ab, and the final Ab was rabbit anti-goat IgG conjugated to alkaline phosphatase (Sigma). All Abs were diluted in PBS/Tween containing 1% BSA. PBS/Tween was used for all plate washes. To detect SC on S-IgA, the final two Ab steps of the assay were modified. The secondary Ab was replaced by rabbit anti-rat SC (a gift from Dr. Brian Underdown, McMaster University, Hamilton, Ontario, Canada), and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) was the final Ab. Since only the influenza-specific mouse IgA could bind to the influenza-coated plates, this assay detected mouse pIgA that had acquired rat SC during the hepatobiliary transport (1).

ELISA binding units

The amount of each purified Ab pool (mIgA, pIgA, or S-IgA) required to give equivalent binding in an IgA ELISA was designated an ELISA binding unit (EBU). One EBU is defined as that dilution of Ab that gives an OD of 1 in an ELISA. Comparison of all three pools was performed in a single assay.

Virus

A/PR8-Mt. Sinai (H1N1) influenza virus was the gift of Dr. Parker Small (University of Florida, Gainesville, FL). To generate a pool, virus was grown in eggs, pooled, filtered through a 0.45-μm pore size Millipore filter, aliquoted, and stored at −70°C. Viral growth was assayed in MDCK cells as described below, and viral titers were calculated by the method of Reed and Muench (16). The $TCID_{50}$ (50% infectious dose in tissue culture) of the virus pool was $10^{5.5}$.

Viral neutralization assay

For neutralization, $10^{2.5}$ $TCID_{50}$ influenza virus was incubated on ice for 1 h in 1, 0.1, 0.01, or 0.001 EBU of purified pIgA or S-IgA anti-influenza Ab or in 10, 1, 0.1, or 0.01 EBU of purified mIgA anti-influenza Ab. Controls included fractionated rat bile and ZF11-15 IgA Ab. To determine neutralization, a modification of the viral assay described by Bender et al. (17) was used. Viral samples were serially diluted (10-fold) in DMEM supplemented with 2.5 μg/ml amphotericin B (Sigma), 50 μg/ml gentamicin, and 10% FCS. Triplicate 100-μl samples of each dilution were placed into 96-well round-bottom tissue culture plates. To each well was added 100 μl of a 2×10^5 cells/ml suspension of MDCK cells in supplemented DMEM/10% FCS. The plates were incubated 24 h at 34°C in 5% CO_2. The culture fluid was removed and replaced with DMEM (150 μl/well) containing 2.5 μg/ml amphotericin B, 50 μg/ml gentamicin, and 2 μg/ml trypsin (DMEM/trypsin). The plates were incubated for 4 days at 34°C in 5% CO_2. Assay for viral growth was by hemagglutination (HA). To each well was added 50 μl of a 0.5% suspension of chicken RBCs (CRBCs). HA was read after 1 to 2 h in the cold. Viral titers were calculated by the method of Reed and Muench (16).

Hemagglutination inhibition

Two HA units (HAU) of influenza virus in 50 μl of PBS were placed in 96-well round-bottom microfilter trays and incubated for 30 min at room temperature with 50 μl of twofold serial dilutions (in PBS) of the appropriate purified IgA pool. Fifty microliters of 0.5% CRBCs were added, and the plates were incubated at 4°C until the pattern could be read (1–2 h). The titer was the $\log_2$ of the last dilution to show complete inhibition of HA.

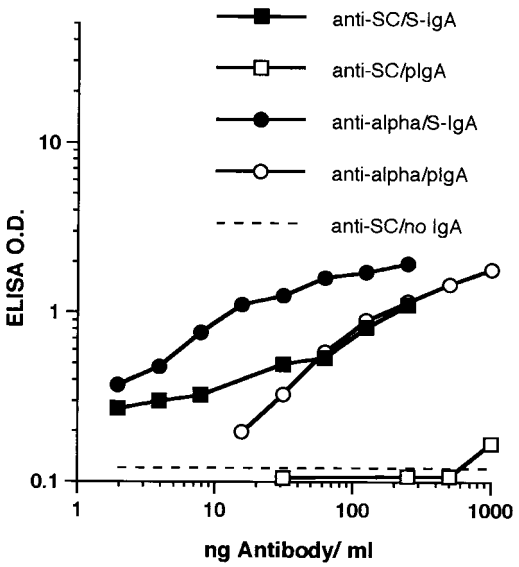

FIGURE 1. Binding of influenza-specific S-IgA and pIgA by anti-rat SC and anti-mouse α antibodies. Purified monoclonal pIgA (open symbols) or S-IgA (closed symbols) was assayed for its ability to bind to influenza virus in an ELISA. Bound Abs were assayed for mouse α-chains (circles) or rat SC (squares) with the appropriate antisera. The dotted line represents the background level of anti-SC binding.

Statistics

Analysis of variance and unpaired two-tailed Student's *t* tests were run with StatView II software (Abacus Concepts, Berkeley, CA) on a MacIntosh Performa 578 computer (Apple Computer, Cupertino, CA). Graphics were generated using CricketGraph software (Computer Associates International, Islandia, NY).

Results

Production of S-IgA

To obtain SC-bearing, mouse influenza-specific IgA, mouse pIgA anti-influenza (H1) mAbs were injected i.v. into a Lewis rat. S-IgA Abs were harvested from the rat bile and assayed by ELISA. The results are shown in Figure 1. Purified mouse anti-influenza pIgA bound to influenza virus and could be detected with anti-mouse α-chain-specific antiserum. It did not contain rat SC; therefore, it could not be detected with anti-rat SC antiserum. Rat bile-passaged mouse anti-influenza pIgA, however, had acquired rat SC during its hepatobiliary transport (1) and could, therefore, be detected by either anti-mouse α or anti-rat SC antiserum. There was a strong correlation ($r = 0.903$) between influenza-specific IgA Ab and SC.

ELISA binding of purified Ab pools

Parallel ELISAs of mIgA, pIgA, and S-IgA were used to calculate the amount of purified IgA in 1 EBU of Ab (Fig. 2). One ELISA binding unit of mouse-rat hybrid S-IgA was equivalent to a 1/30 dilution of the purified pool, while 1 EBU of pIgA was equivalent to a 1/2400 dilution and 1 EBU of mIgA was equivalent to a 1/800 dilution of the respective Ab pool. One ELISA binding unit of pIgA or S-IgA contained ~170 ng of Ab while 1 EBU of mIgA contained ~190 ng of Ab.

Biologic activity of mIgA vs pIgA Abs

To determine whether p-Ig are more efficient than m-Ig in biologic activity, the abilities of pIgA and mIgA to inhibit influenza-mediated HA of CRBC and to neutralize viral infectivity were compared. Inhibition of 2 HAU of influenza virus (Fig. 3) required 1.72 ± 0.86 EBU of mIgA, while only 0.33 ± 0.22 EBU of pIgA

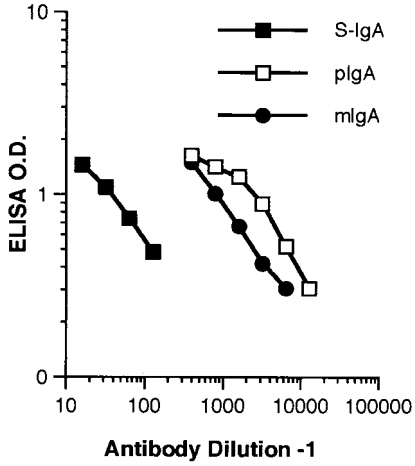

FIGURE 2. ELISA titers of pIgA, S-IgA, and mIgA anti-influenza mAbs. Purified S-IgA (closed squares), pIgA (open squares), and mIgA (closed circles) were tested simultaneously for their ability to bind to influenza virus in an ELISA. One ELISA binding unit of each preparation was calculated as the Ab dilution that gave an OD of 1 in this assay.

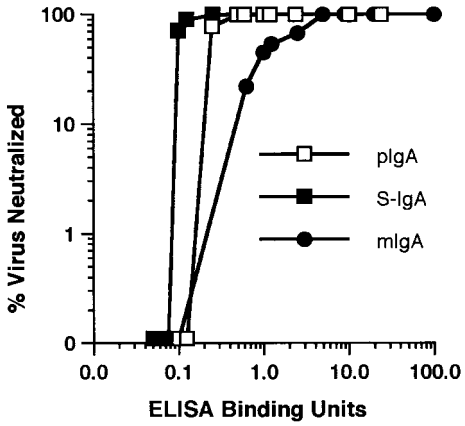

FIGURE 4. Neutralization of PR8 influenza virus by pIgA, S-IgA, and mIgA anti-H1 mAbs. Various dilutions of purified pIgA (open squares), S-IgA (closed squares), or mIgA (closed circles) were incubated on ice for 1 h with $10^{2.5}$ $TCID_{50}$ PR8 influenza virus, then 10-fold serial dilutions of the neutralization mixture were assayed on MDCK cells using the standard neutralization protocol. Fractionated normal rat bile and ZF11-15 IgA Ab, which were included as controls, did not neutralize virus.

was required for the same degree of inhibition. This is a significant difference ($p = 0.05$), with pIgA being more than 5 times more efficient than mIgA in inhibiting viral HA.

The results of the comparison of the abilities of pIgA and mIgA to neutralize viral infectivity (Fig. 4) agree with the HAI data. Neutralization of 50% of the viral infectivity required 1.1 EBU of mIgA, but only 0.15 EBU of pIgA. Thus, pIgA is approximately 7 times more efficient than mIgA in neutralizing viral infectivity.

Biologic activity of pIgA vs S-IgA

To determine whether the addition of SC enhanced the biologic activity of pIgA, the abilities of pIgA and S-IgA to inhibit viral HA and neutralize viral infectivity were compared. Inhibition of the activity of 2 HAU of influenza virus (Fig. 3) required 0.33 ± 0.22 EBU of pIgA or 0.26 ± 0.15 EBU of S-IgA (not significant by Student's *t* test). The addition of SC, then, did not enhance HI.

The viral neutralization data agreed with the HI data. Neutralization of 50% of the viral infectivity required 0.15 EBU of pIgA or 0.09 EBU of S-IgA; thus, S-IgA is only marginally (1.4 times) more effective than pIgA in neutralizing influenza virus. Normal rat bile or nonspecific murine pIgA did not neutralize viral infectivity.

Functional stability of S-IgA vs mIgA or pIgA

Although the addition of SC to pIgA does not greatly modify its in vitro biologic activity, its functional activity in vivo may be enhanced by this addition due to the stability conferred upon the molecule by SC-mediated protection of protease sites (8, 9). To test the functional stability of S-IgA, pIgA, or mIgA, Ab was incubated overnight at 34°C in 0.0002% trypsin, then tested for its ability to neutralize virus (Fig. 5). The virus-neutralizing activity of all concentrations of S-IgA was unaffected by trypsin preincubation. One and ten ELISA binding units of pIgA were unaffected by trypsin treatment; however, 0.1 EBU (17 ng/ml) showed a 67% reduction in activity following trypsin treatment. Although this

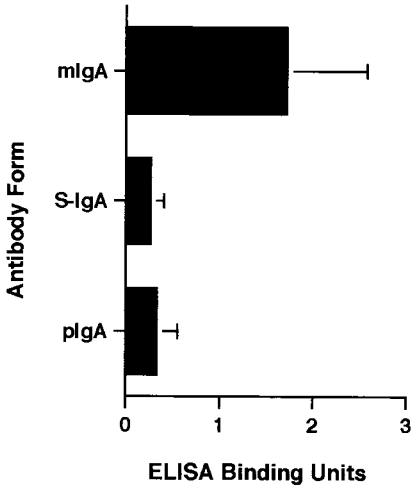

FIGURE 3. Hemagglutination inhibition by pIgA, S-IgA, and mIgA. PR8 influenza virus (2 HAU) was incubated for 30 min at room temperature with varying amounts of purified pIgA, S-IgA, or mIgA, then mixed with 0.5% CRBCs and incubated on ice until an HA pattern was apparent.

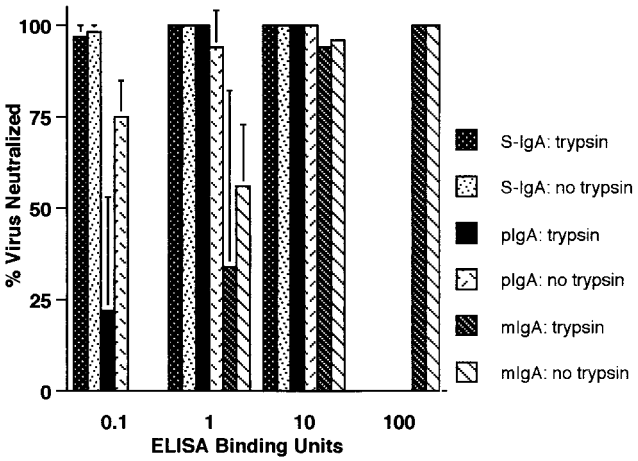

FIGURE 5. Effect of trypsin preincubation. S-IgA or pIgA (0.1, 1, or 10 EBU) or mIgA (1, 10, or 100 EBU) was preincubated overnight at 34°C in the presence or the absence of 0.0002% trypsin, then incubated on ice for 1 h with $10^{2.5}$ $TCID_{50}$ PR8 influenza virus. Tenfold serial dilutions of the neutralization mixture were assayed on MDCK cells using the standard neutralization protocol.

reduction was not statistically significant ($p = 0.143$, by analysis of variance), it does support the concept of enhancement of the functional stability of pIgA by SC addition. The activity of mIgA was the most sensitive to trypsin, with 1 EBU undergoing a significant ($p = 0.011$) loss of virus-neutralizing activity following preincubation.

Discussion

Addition of SC confers increased stability on the resultant S-IgA (8, 9), apparently by helping to hold the IgA monomers together (9) and by masking proteolytic sites from proteases present in mucosal secretions (8); however, the effect of the addition of SC on the biologic activity of the IgA Fab is unknown. It has been suggested (10) that SC might augment the ability of IgA to neutralize pathogens. To study the effect of the addition of SC, it was necessary to produce S-IgA and pIgA specific for the same epitope. Obtaining sufficient quantities of purified specific murine S-IgA and demonstrating that the IgA contained SC presented a problem, since there was no antiserum to mouse SC available. To overcome this difficulty and produce pools of murine pIgA and S-IgA of the same specificity, mouse pIgA monoclonal anti-influenza Ab was injected i.v. into rats, the bile duct was cannulated, and bile was collected. Polymeric Igs undergo hepatobiliary transport in rats and mice, acquiring SC as they are transported through hepatocytes (1, 18); thus, the rat bile contained mouse pIgA bearing rat SC and specific for influenza virus. This S-IgA bound both influenza virus and rabbit anti-rat SC in an ELISA and had the anti-influenza specificity of the injected pIgA. When comparable amounts of pIgA and S-IgA were assayed for biologic activity, S-IgA and pIgA were equally effective in inhibiting viral hemagglutination, and S-IgA was only marginally (1.4 times) more effective than pIgA in viral neutralization. Hence, the addition of SC does not enhance the biologic activity of pIgA in vitro. Trivial explanations for the failure of SC to modify the in vitro function of pIgA include the possibilities that 1) only a small proportion of the IgA molecules obtained from the bile actually contained SC, and 2) rat SC does not function as efficiently with murine IgA as mouse SC does. It has been shown (11) that bile (i.e., S-IgA) from mice passively immunized with the same monoclonal pIgA as that used in the current study is no more effective than pIgA in neutralizing influenza virus; hence, it is unlikely that the failure of SC addition to modify in vitro Ab function is due to a lack of cooperation between mouse IgA and rat SC. It is also unlikely that only a small proportion of biliary IgA actually contains SC. Hepatobiliary pIgA secretion correlates with hepatocyte expression of SC (19). It has been demonstrated (19–22) that the transport of pIgA in the rat is dependent upon SC; non-SC-bearing pIgA is not found in significant quantities in bile. While other IgA binding sites may exist on hepatocytes, it is unlikely that Ab bound at these sites would reach the bile as an intact, functional molecule (23). The mouse-rat hybrid Ab measured in our ELISA contains a functional Fab, since it retains the capacity to bind virus in the assay; it is likely that it also contains J chain, since J chain is required for SC binding (1, 18), and an intact Fc region, since Cα2 and Cα3 are involved in the binding of SC (1, 18). The SC content of our hybrid Ab closely correlates ($r = 0.903$) with its influenza-specific IgA content.

Lindh (8) demonstrated that binding of SC increases the resistance of pIgA to proteolysis. We studied the effects of preincubation of S-IgA, pIgA, or mIgA in 0.0002% trypsin on virus-neutralizing activity. This trypsin level was chosen because it is the concentration of trypsin required for the growth of influenza virus, a mucosal pathogen, in vitro. The activity of S-IgA was unaffected by trypsin preincubation, as was the activity of higher concentrations of both pIgA and mIgA. At concentrations of 17 or 190 ng/ml, respectively, trypsin preincubation decreased the ability of pIgA and mIgA to neutralize virus. Monomeric IgA was the most sensitive to proteolysis, with a significant loss of activity at an Ab concentration of 190 ng/ml. At a concentration of 17 ng/ml, the neutralizing ability of pIgA was reduced by 67%; however, pIgA activity was unaffected at an Ab concentration of 170 ng/ml. These data suggest that the functional activity of pIgA is up to 10 times more resistant to proteolysis than that of mIgA and that the activity of S-IgA is up to 10 times more resistant to proteolysis than that of pIgA. This finding is consistent with the hypothesis (1, 18) that binding of SC masks proteolytic sites on the pIgA molecule and also suggests that dimerization by the addition of J chain may block proteolytic sites on the α-chain of the IgA monomer as well. Thus, even though the addition of SC does not enhance the activity of the Fab in vitro, it does confer additional functional stability upon the S-IgA molecule in vivo.

Taylor and Dimmock (24) showed that the mechanism of neutralization of influenza virus by S-IgA was different from that of monomeric Igs. Monomeric IgA apparently allows normal attachment, uncoating, and migration of the viral genome into the cell nucleus, with blocking of virus growth at some point after entry into the nucleus. In contrast, pIgA (25) can neutralize influenza virus by one of three mechanisms, depending upon Ab concentration. With high concentrations of pIgA or S-IgA, virus is 1) prevented from attaching to cells, or if it does attach, it is 2) not internalized; with low concentrations of Ab, virus may 3) attach and be internalized, but it is unable to carry out the fusion event needed for uncoating. The variety of mechanisms by which pIgA can neutralize influenza suggests that pIgA would be much more efficient than mIgA in virus neutralization.

Previous work (11–13) (see Footnote 5) has indicated that pIgA is 5 to 10 times more effective than m-Ig in neutralizing viruses; however, the monomer used has generally been IgG. No quantitation of activity has been performed with monoclonal mIgA and pIgA of matched specificity, although Taylor and Dimmock (24) compared the activity of polyclonal S-IgA purified from rat bile with that of its chemically dissociated monomer. The definitive experiment would compare pIgA with mIgA having the same Fab, thus eliminating variables related to class, Ab treatment, or specificity; however, this experiment would involve the production of "engineered" Ab molecules. Dissociation of BALB/c pIgA to provide monomers is not feasible, since this molecule, being similar to human IgA2, is unstable to chemical manipulation. Although they are both directed toward the H1 hemagglutinin of influenza virus (14), the mIgA and pIgA mAbs used in this study have not been shown to have identical binding sites; thus, they satisfy only two of the three criteria. They do, however, have similar binding characteristics, as measured by ELISA activity. We determined how much mIgA and pIgA were required to produce equal binding to influenza in an ELISA (equivalent EBUs). One ELISA binding unit of Ab activity contained approximately the same amount of mIgA or pIgA (190 vs 170 ng, respectively) or roughly twice as much mIgA as pIgA on a molar basis. We found that, on a weight to weight basis, pIgA is 5 times more effective in inhibiting viral hemagglutination and ~7 times more effective in viral neutralization (standard viral neutralization assay) than mIgA. This is in agreement with the published data for comparisons of IgG and pIgA. Thus, the determining factor in neutralization efficiency is apparently the molecular form of the Ab, i.e., monomer vs polymer, and not the isotype of the monomeric Ig, and pIgA is, within the limitations of our study, more effective than mIgA in neutralizing influenza virus. In addition, the functional activity of pIgA is up to 10 times more stable than that of mIgA in a degradative

environment, making pIgA up to 70 times more effective than monomeric Ab in vivo. Thus, if protective Ab is to be administered passively to, for example, respiratory mucosal surfaces by intranasal installation, both monomeric and polymeric forms should be effective; however, pIgA protection should require less Ab and be longer lasting than mIgA protection.

The finding that pIgA is more effective than mIgA in neutralizing influenza virus acquires additional biologic significance upon consideration of the anatomical site of S-IgA action. Polymeric IgA is produced by plasma cells adjacent to the mucosal epithelial cells where infection occurs (1, 26). It has been shown that during its transcytosis through these epithelial cells to become S-IgA, pIgA may neutralize intracellular virus (27). Thus, mucosal IgA provides two barriers to infection: extracellular S-IgA and intracellular pIgA. Immunization efforts should consider, in addition to monomeric Abs in the plasma, the induction of S-IgA to prevent viral infection of mucosal surfaces.

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