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J Immunol (1980) 124 (6): 2620–2624.
https://doi.org/10.4049/jimmunol.124.6.2620

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0022-1767/80/1246-2620$02.00/0
THE JOURNAL OF IMMUNOLOGY
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Vol. 124, No. 6 June 1980
Printed in U.S.A.

VARIABLES AFFECTING THE LOCAL IMMUNE RESPONSE IN THIRY-VELLA LOOPS

II. Stability of Antigen-Specific IgG and Secretory IgA in Acute and Chronic Thiry-Vella Loops[1]

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The predominant mucosal immune response to antigens administered into Thiry-Vella loops in rabbits has been IgA with little or no antigen-specific IgG detected in the intestinal secretions. However, there has been no evidence that antigen-specific IgG could be recovered at all if IgG were being secreted in response to the locally administered antigen. In the present study, solutions containing either IgG or IgA activity against a *Shigella flexneri-Escherichia coli* hybrid (shigella X16) were incubated *in vivo* in Thiry-Vella loops of 10 rabbits and *in vitro* at 37°C in pooled freshly collected intestinal secretions. The volume of fluid recovered from chronic Thiry-Vella loops was significantly decreased after 10 min or longer of *in vivo* incubation. After 100 min of *in vivo* incubation an average of 50% of the antigen-specific IgA activity and 42% of the antigen-specific IgG activity could be recovered. Complete recovery of both the IgA and IgG activity was achieved in the *in vitro* incubations. Gel diffusion studies demonstrated that the IgA and IgG activity remaining after 100 or 200 min of *in vivo* incubation was in the m.w. range of intact dimeric secretory IgA and intact monomeric IgG, respectively. These studies indicate that the stability of both IgG and secretory IgA in the lumen of Thiry-Vella loops is sufficient to allow their detection by sensitive immunochemical techniques.

Although it is generally accepted that the secretory (local) IgA response of mucosal surfaces is important in defense against various microorganisms and their toxic products, the kinetics of the establishment of this response is only incompletely understood. One major problem in studying local (especially enteric) immune responses has been the difficulty involved in collecting sequential mucosal secretions after local stimulation with antigen.

Received for publication December 19, 1979.
Accepted for publication February 28, 1980.
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[1] This work was supported in part by University of Michigan Biomedical Research Council Grant 016153 and by Horace H. Rackham Grant 387455.
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Recently, several groups have developed chronically isolated (Thiry-Vella) loop models that have been used to study the local immune response in several different species (1–6). In our own rabbit Thiry-Vella loop model system we and others who have used our model have shown that these loops can mount a vigorous secretory IgA response to such diverse antigens as cholera toxin, choleragenoid, KLH, DNP-KLH, and live bacteria (7–11). However, in all these studies only trivial amounts or no IgG antibodies against the specific antigens used were found in the loop secretions (7–11).

It has been unclear whether this lack of specific IgG in intestinal secretions in our studies is due to rapid degradation of this antibody in the isolated intestinal loop or to lack of formation of specific IgG after local immunization, since others have found that IgG can transudate into the intestine from the serum (12). The present study compares the recovery of intact antigen-specific secretory IgA with that of intact antigen-specific IgG after incubation in both acute and chronic intestinal loops and *in vitro* in fresh loop fluids.

MATERIALS AND METHODS

Preparation and care of Thiry-Vella loops. Our previously described method (3) was used to construct chronically isolated segments of ileum, 20-cm long in 10 2 to 3 kg New Zealand white rabbits. Briefly, while the animals were anesthetized with a combination of xylazine and ketamine, a midline abdominal incision was made, the terminal ileum was identified, and a 20-cm segment containing a single Peyer's patch was isolated with its vascular supply intact. An end-to-end anastomosis restored continuity to the ileum. Silastic tubing was sewn into each end of the 20-cm segment of ileum. The silastic tubing was brought out through the midline abdominal incision and tunnelled subcutaneously to an opening in the nape of the neck where it was secured.

Secretions from the isolated loops were collected daily by injecting 20 ml of air into one end of the silastic tubing thereby expelling loop fluid from the other silastic tube. Approximately 2 to 4 ml of fluid were obtained daily from each loop. This fluid was centrifuged at 3500 rpm for 10 min on a Sorvall GLC-3 centrifuge to separate mucus and cell debris. The clear to translucent supernatant was stored at −20°C. After the collection of specimens, the loops were flushed with saline to prevent mucus from obstructing the silastic tubes. This saline was flushed from the loop by repeated injections of air.

Antibody preparations. Antiserum with IgG antibodies to shigella X16 (IgG anti-X16) were kindly provided by Dr. S. B. Formal. They were stimulated by repeated i.v. injection of 10^6

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heat-killed shigella X16 into rabbits. The IgG anti-X16 activity was determined by use of the enzyme-linked immunosorbent assay (ELISA)[3] (13).

IgA antibodies to shigella X16 (IgA anti-X16) were raised by repeated immunization of Thiry-Vella loops in rabbits with 10^8 live shegella X16 as previously described (8). Several loop secretions were pooled and the IgA anti-X16 activity was determined by ELISA (13). Neither IgG nor IgA anti-X16 activity was found in intestinal secretions from unimmunized rabbits.

ELISA procedure. ELISA for IgA and IgG activity to shigella X16 lipopolysaccharide (X16 LPS) was performed as previously described (13). All reactions were performed in duplicate. This technique allows detection of as little as 1.3 ng of specific antibody per milliliter (13). Coefficients of variation for replicate samples of IgG and IgA anti-X16 were 3.6 and 9.0%, respectively.

In vivo immunoglobulin recovery. Rabbits with chronically isolated Thiry-Vella loops for 3 to 5 weeks were anesthesized with xylazine and ketamine. A midline abdominal incision was made and the loops were inspected visually. In order to minimize dead space, the silastic tubing was trimmed such that only 5 cm protruded from the loop. At this time a second 20-cm segment of ileum with a Peyer's patch (acute loop) was created with a short 5-cm length of silastic tubing attached to each end. The purpose of the acute loop was to determine whether isolation of the chronic loop changed the ability to recover fluid volume or specific immunoglobulins.

Three or 4 ml of the IgG or IgA anti-X16 preparations (as indicated in each study) were added to both the acute and chronic loops. Both silastic tubes were clamped off close to the cuffs of the isolated loops, which further decreased the dead space and prevented leakage of fluid. After 10 min the clamps were removed and air was injected into one of the tubes. The fluid expelled from the other tube was collected, the volume was measured, and then stored at −20°C for determination of specific activity at a later date. The above procedure was repeated with the IgG or IgA anti-X16 preparations being left in the acute and chronic loops for 50, 100, and 200 min.

IgG and IgA anti-X16 activity remaining in each aliquot after incubation in the acute and chronic loops was determined by ELISA.

Immunoglobulin recovery in pooled loop secretions. Fresh loop secretions (containing mucus, debris, and live bacteria) from several unimmunized rabbits were pooled and mixed with an equal volume of the IgG or IgA anti-X16 preparations. These mixtures were incubated at 37°C, with samples removed at 10, 50, 100, and 200 min, and stored at −20°C. The IgG or IgA anti-X16 activity remaining in these samples were determined by ELISA.

Gel filtration. Samples incubated for 100 and 200 min in acute and chronic loops of several rabbits were pooled. Two milliliters of these or of the IgG or IgA anti-X16 preparations were dialyzed against 0.1 M Tris-HCl saline buffer, pH 7.5, and applied to a column (2.5 x 96 cm) of Sephadex G-200 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) that had been equilibrated in the same buffer at 4°C. Chromatography was performed with reverse flow at 12 to 15 ml/hr. Five-milliliter fractions were collected and monitored with a Beckman Model 35 spectrophotometer (Beckman Instruments, Irvine, Calif.) at 280 nm. Fractions were pooled as indicated and concentrated 10-fold by an ultrafiltration cell (Amicon Corporation, Lexington, Mass.). IgG or IgA anti-X16 activity in each concentrated fraction was determined by ELISA. The G-200 column was calibrated by using ribonuclease A (m.w. 13,700), chymotrypsin A (m.w. 25,000), ovalbumin (m.w. 45,000), aldolase (m.w. 158,000), catalase (m.w. 232,000), ferritin (m.w. 440,000), and thyroglobulin (m.w. 669,000) (Pharmacia Fine Chemicals, Inc.).

[3] Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; AP-GARG, alkaline phosphatase-conjugated goat anti-rabbit IgG; AP-GARA, alkaline phosphatase-conjugated goat anti-rabbit IgA; IgG or IgA anti-X16, IgG or IgA against shigella X16 lipopolysaccharide; PTA, phosphate-buffered saline with Tween 20 and sodium azide.

RESULTS

Fluid volume recovery from acute and chronic loops. The volume of fluid recovered after each incubation period is listed in Table I. It is apparent that considerably less fluid was recovered from the chronic than from the acute loops. A significant decrease in fluid recovered in chronic loops was found at 10 min ($p < 0.01$) or longer.

After the fluid volume data were obtained from the first animal we were concerned about specific antibody either being absorbed by or adhering to the mucosal surfaces. In an attempt to recover any antibodies adhering to the surface of the gut lumen, a wash solution consisting of from 3 to 4 ml of PBS (as indicated) was instilled into each loop and flushed out (under direct vision) with large volumes of air. The volume of wash solution recovered was measured and aliquots were stored at −20°C for specific antibody assay by ELISA. Total recovery of the wash solution was obtained in both the acute and chronic loops (data not shown).

In vivo IgG and IgA recovery. Specific IgA and IgG anti-X16 activity recovered from both incubated preparations and in the wash fluids in acute and chronic loops is listed in Tables II and III. The apparent increase in IgA activity in some of the 100- and 200-min samples from the chronic loop (Table II) likely reflects increased absorption of fluids by chronic loops (Table I) with resultant concentration of the IgA anti-X16 activity. That this was the case is further demonstrated by the presence of greater antibody activity in the chronic loop wash specimens than in the acute loop wash specimens at 100 and 200 min (Table II).

To compare the ability of IgG and IgA anti-X16 to remain intact in the chronic and acute loops, the percent specific activity recovered was computed by the formula:

$$\% \text{ Recovery} = \frac{(I \times Vi) + (W \times Vw) \times 100}{(C \times Vc)}$$

where I = incubated fluid ELISA value, Vi = volume of incubated fluid recovered, W = wash fluid ELISA value, Vw = volume of wash fluid recovered, C = ELISA value of control fluid instilled into loop, Vc = volume of control fluid given.

As shown in Table IV, most of the IgA anti-X16 activity could be recovered at all the intervals examined. Recovery was better in the acute loops than in the chronic loops. This likely

TABLE I

Volume of fluid recovered from isolated loops

Loop	Volumes Given[a]	Volume Recovered[b] at			
		10 min	50 min	100 min	200 min
Acute	3.15 ± 0.10	2.84 ± 0.11	2.67 ± 0.12	3.07 ± 0.10	3.31 ± 0.10
Chronic	3.15 ± 0.10	2.26 ± 0.14	1.93 ± 0.16	1.11 ± 0.12	0.52 ± 0.09

[a] Mean volume (ml) ± S.E.M. of IgG or IgA anti-X16 given before each time period.

[b] Mean volume (ml) recovered by repeated air flush under direct vision until no more fluid could be collected.

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TABLE II

IgA activity in incubated fluids and wash fluids from acute and chronic loops

Rabbit	Loop	Control[a]	10[b] Min	10-Min Wash	50 Min	50-Min Wash	100 Min	100-Min Wash	200 Min	200-Min Wash
M1	Acute	3.497[c]	3.943	N.D.[d]	4.797	N.D.	4.797	N.D.	4.598	N.D.
M1	Chronic	3.497	5.990	N.D.	4.939	N.D.	6.094	N.D.	7.694	N.D.
M2	Acute	1.138	1.863	0.150	1.382	0.193	1.325	0.084	1.207	0.229
M2	Chronic	1.138	1.418	0.235	1.223	0.283	2.762	0.554	1.387	0.443
M3	Acute	1.607	1.228	0.285	0.674	0.130	0.466	0.202	0.523	0.132
M3	Chronic	1.607	0.715	0.131	0.657	0.338	0.608	0.204	1.210	0.336
M4	Acute	1.242	0.882	0.293	0.838	0.415	0.604	0.318	0.630	0.230
M4	Chronic	1.242	0.796	0.328	1.117	0.373	0.709	0.506	1.263	0.725
M5	Acute	0.968	0.901	0.174	0.598	0.105	0.543	0.084	0.777	0.023
M5	Chronic	0.968	0.552	0.147	0.587	0.282	1.366	0.341	1.241	0.489

[a] IgA anti-X16 value determined by ELISA of preparation given to each animal.
[b] Loop fluid and wash fluid samples at indicated times after instillation of antibody into loops.
[c] Final dilution of IgA anti-X16 for M1 was 1:160 and for M2-M5 was 1:320.
[d] N.D., not done.

TABLE III

IgG activity in incubated fluids and wash fluids from acute and chronic loops

Rabbit	Loop	Control[a]	10[b] Min	10-Min Wash	50 Min	50-Min Wash	100 Min	100-Min Wash	200 Min	200-Min Wash
M7	Acute	0.659[c]	0.392	0.060	0.168	0.027	0.274	0.038	0.151	0.044
M7	Chronic	0.659	0.295	0.045	N.D.[d]	0.055	N.D.	0.094	N.D.	0.137
M8	Acute	0.982	0.446	0.044	N.D.	0.054	0.267	0.067	0.529	0.098
M8	Chronic	0.982	0.596	0.054	N.D.	0.081	0.752	0.208	N.D.	0.425
M9	Acute	0.693	0.290	0.050	0.581	0.110	0.472	0.066	1.031	0.088
M9	Chronic	0.693	0.864	0.046	0.171	0.102	0.210	0.094	0.831	0.102
M10	Acute	1.031	0.267	0.037	0.229	0.078	0.202	0.081	0.399	0.036
M10	Chronic	1.031	0.132	0.043	0.240	0.076	0.323	0.093	0.221	0.072
M27	Acute	2.927	0.925	0.450	1.140	0.407	1.080	0.454	1.247	0.322
M27	Chronic	2.927	1.389	0.136	1.465	0.192	1.262	0.212	1.597	0.470

[a] IgG anti-X16 value determined by ELISA of preparation given to each animal.
[b] Loop fluid and wash fluid samples at indicated times after instillation of antibody into loops.
[c] Final dilution of IgG anti-X16 for M7-M10 was 1:1200 and for M27 was 1:320.
[d] N.D., not done.

reflected antibodies adhering to the lumenal surface to a greater extent in the chronic loops than in the acute loops since the wash fluids from the chronic loops had greater IgA anti-X16 activity than those from the acute loops.

Table IV demonstrates that although IgG anti-X16 activity was recoverable from the isolated loops, the recovery was less consistent than that of IgA anti-X16. As with IgA anti-X16 the recovery of IgG anti-X16 was somewhat better in the acute loops than in the chronic loops.

Gel chromatography of IgG and IgA anti-X16. The ELISA on concentrates of the IgA anti-X16 control pooled fractions demonstated that most of the IgA anti-X16 activity had a m.w. in the range of approximately 350,000 to 650,000. Some IgA anti-X16 activity was present in the 12,000 to 24,000 m.w. range. Most of the IgA anti-X16 recovered in fluids incubated in both acute and chronic loops for 100 and 200 min was present in the 350,000 to 560,000 m.w. with a small amount of activity present in the 12,000 to 24,000 m.w. range (Fig. 1).

The ELISA on individual fractions from the elution profile for the IgG anti-X16 control preparation indicated that most of the IgG anti-X16 activity was in the m.w. range of 140,000 to 200,000. As with the IgA anti-X16, some IgG anti-X16 activity was present in smaller fragments as well. Most of the IgG anti-X16 activity recovered in both acute and chronic loops for 100 and 200 min was in the molecular weight range of intact IgG (Fig. 2).

TABLE IV

Percentage of antibody recovery

Loop	10 Min	50 Min	100 Min	200 Min
	IgA Recovery			
Acute	94 ± 2.8[a]	79 ± 9.8	76 ± 7.5	87 ± 5.6
Chronic	62 ± 10.7	68 ± 9.6	50 ± 6.2	51 ± 5.2
	IgG Recovery			
Acute	48 ± 5.3	43 ± 5.9	42 ± 5.0	55 ± 7.2
Chronic	50 ± 11.6	33 ± 3.1	42 ± 12.3	27 ± 7.4

[a] Values are percentage of mean ± S.E.M. See text for calculation.

In vitro IgA and IgG recovery in loop secretions. Because of the difficulty in recovering fluid from the chronic loops, *in vitro* studies of IgG and IgA stability were performed to control for

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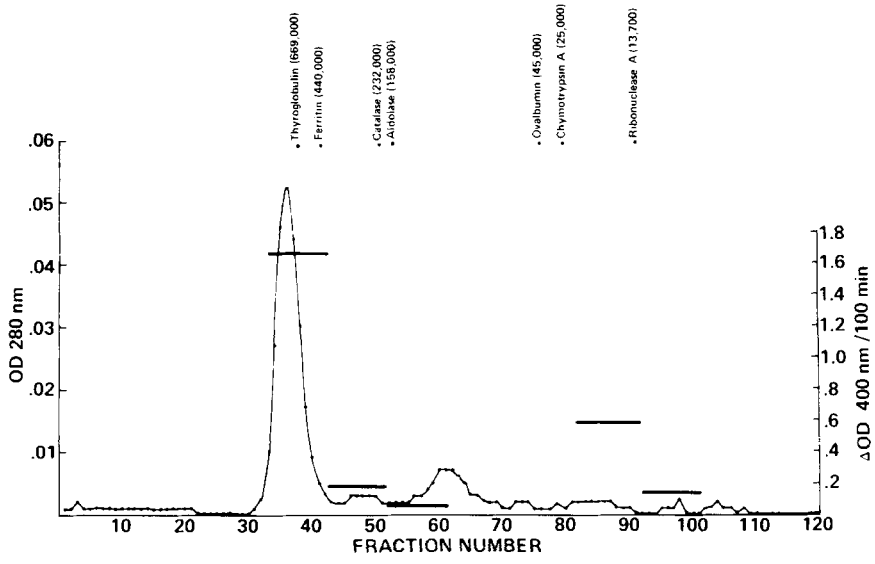

Figure 1. Elution profile of IgA anti-X16 activity in pooled loop secretions incubated for 200 min in acute Thiry-Vella loops. O.D. 280 nm of each fraction indicated on left ordinate (●). IgA anti-X16 activity in concentrated pooled fractions on right ordinate (——). Position of m.w. indicators shown.

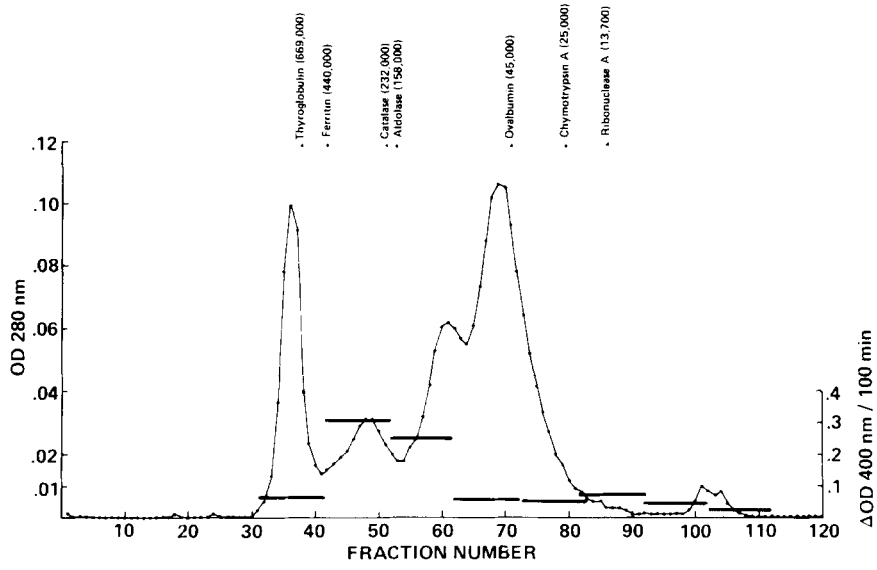

Figure 2. Elution profile of IgG anti-X16 activity in pooled positive serum incubated for 200 min in acute Thiry-Vella loops. O.D. 280 nm of each fraction indicated on left ordinate (●). IgG anti-X16 activity in concentrated pooled fractions on right ordinate (——). Positions of m.w. indicators shown.

fluid loss and antibody adsorption to surface epithelium.

Several experiments with different concentrations of IgA and IgG anti-X16 incubated *in vitro* with fresh loop fluid at 37°C had identical results. Two representative studies are shown in Table V. Virtually all the IgA and IgG anti-X16 activity was recovered after incubation for 10, 50, 100, and 200 min in fresh pooled loop fluid at 37°C.

TABLE V

IgA and IgG activity in loop fluid in vitro

Sample	IgA Activity[a]	IgG Activity[b]
Control	1.742	1.712
10-min loop	1.773	2.209
50-min loop	1.854	2.125
100-min loop	2.258	2.035
200-min loop	2.066	2.122

[a] ELISA activity of 1:320 final dilution of IgA anti-X16 incubated in fresh pooled loop fluid at 37°C for the indicated times.

[b] ELISA activity of 1:400 final dilution of IgG anti-X16 incubated in fresh pooled loop fluid at 37°C for the indicated times.

DISCUSSION

Chronically isolated segments of intestine in several species have been used to demonstrate that the predominant mucosal immune response to locally administered antigen is secretory IgA (1–10). Although this was consistent with the generally accepted notion that the local humoral immune response is due mainly to secretory IgA, it was not clear whether, if produced, antigen-specific IgG antibodies could be recovered in detectable amounts from the isolated loop secretions.

Several studies have demonstrated that secretory IgA is more stabile in a proteolytic environment than IgG or IgA without secretory component (14–17). Much of the increased resistance of secretory IgA to enzymatic attack is attributed to the presence of secretory component (15–17). Recent data suggest that secretory component is bound covalently to one of the α-chains in human secretory IgA (18).

All previous studies on stability of immunoglobulins to proteolytic degradation were conducted under *in vitro* conditions that allowed careful control of variables (14–16). The present study sought to determine the stability of specific IgG and specific secretory IgA molecules by placing them in isolated segments of intestine where the immunoglobulins would be subject to several degradative processes, but which may better

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reflect the complex *in vivo* situation in such isolated loops. For instance, the chronically isolated segments of intestine in our rabbit Thiry-Vella loops model have been shown to undergo atrophy of the villi and hypertrophy of the the crypts with Paneth cell hyperplasia (3). Further, these Thiry-Vella loops become colonized with *Pseudomonas aeruginosa* and other bacteria that may adversely affect the intralumenal antibodies (19).

Some problems were encountered in trying to recover specific antibody activity. One unexpected finding was that the chronically isolated loops absorbed fluid to a greater extent than the acutely isolated loops. This increased absorption of fluid by the chronic loop reflects the physiologic correlate of previously described morphologic changes (3). As a result, at the 100- and 200-min intraloop incubation rather little fluid was available for assay in many of the chronic loop studies. By performing a wash procedure after each incubation period, we were able to recover considerably more antibody at these time periods. This indicated that although some loss of antibody may have occurred, much of the difficulty in recovering antibody activity was likely due to antibody adhering to the surface epithelium or glycocalyx.

Despite these problems, we were able to recover much of the antigen-specific IgA and IgG from all of the rabbit loops studied. Although the recovery of secretory IgA was somewhat better than that for IgG, gel diffusion studies demonstrated that the antigen-specific IgA and IgG recovered from both the acute and chronic loops were predominantly intact molecules. If proteolytic digestion of these immunoglobulins is occurring in these isolated loops the rate of such degradation must be quite slow.

Because of the difficulties in recovering fluid volumes from the chronically isolated loops, the stability of antigen-specific IgA and IgG in fresh pooled loop fluid *in vitro* was studied. The fresh pooled loop fluid used contains mucus, cell debris, and large numbers of bacteria, yet no decrease in antigen reactivity of IgA or IgG was found after as long as 200 min of incubation at 37°C *in vitro*.

The good recoverability of intact antigen-specific IgG in both the *in vitro* and *in vivo* systems wasn't unexpected. Although some pancreatic enzymes may remain in the glycocalyx (20) of these isolated loops, the lumen of these loops is separated from continuous exposure to gastric acid, bile, or pancreatic enzymes (3). Also, the dipeptidases present in intestinal epithelial cells would not be expected to have a significant effect on antibody stability.

In conclusion, the previous studies showing a strong antigen-specific IgA response with no, or only trivial, antigen-specific IgG likely reflected the *in vivo* functions of the secretory immune system in intestinal segments. The present study demonstrates that antigen-specific IgG and secretory IgA are not rapidly degraded in the environment of either the acute or chronic isolated ileal loops in rabbits. Although not as recoverable as secretory IgA in the lumen of Thiry-Vella loops, if IgG was a substantial part of the local immune response, its stability was sufficient to allow its detection by sensitive immunochemical methods.

Acknowledgments. The authors would like to thank Dr. S. B. Formal, Dr. P. Gemski, and Mr. H. H. Collins for providing reagents and advice and Ms. Debbie Joseph, Mrs. ZoAnn Biddle, and Mrs. MaryAnn Byrnes for their assistance in preparing this manuscript.

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