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SUSCEPTIBILITY OF MONOCLONAL IGG PARAPROTEINS TO PLASMIC CLEAVAGE USING GLYCERIN-STABILIZED HUMAN PLASMIN

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To shed further light on plasmin-IgG interactions a simple procedure is described that permitted 48 monoclonal IgG isolates from human serum to be profiled for their susceptibility to plasmic cleavage. In addition to anodal Fc and cathodal Fab fragments, combined immunoelectrophoresis-electrophoresis revealed transient anodal banding, as well as Fab-fragment subcleavage in many of the IgG subclass-1 isolates. The subcleavage of released Fab fragments, which bear the idiotype determinants, points to a possible ancillary role of plasmin in "idiotype processing" leading to immunoregulatory anti-idiotype networks. The cleavage of IgG by endogenous plasmin also points to a possible active role of plasmin in the "steady-state" metabolism of IgG.

The rapid autolysis of plasmin in the inhibitor-free solutions needed to study its uninhibited endopeptidase activity has undoubtedly limited its wider use as an investigative enzyme, as compared, for example, with extensive use of the latex-derived enzyme, papain, for studying the structural subcomponents of IgG (1). A recent study of plasminogen-plasmin-IgG interactions by Harpel et al. (2), however, has indicated that physiologic-range concentrations of fluid-phase plasminogen can form activator-susceptible complexes with polyclonal IgG leading to IgG cleavage. This study describes a simple procedure, utilizing glycerin-stabilized human plasmin (3), that permitted quantifiable profiling of 48 monoclonal IgG isolates from human serum for their susceptibility to direct cleavage by fluid-phase plasmin after: overnight, 2-day and several day (i.e. 6 or 7 day) incubation at room temperature. Utilization of this procedure for more definitive studies could shed much further light on the physiological implications of plasminogen-plasmin-IgG interactions in respect to such questions as: 1) the selective digestion of antigen-antibody complexes and other "misfolded" proteins in the circulation (4), 2) the up-regulation of B-lymphocyte proliferation via a plasmin-generated Fc fragments of IgG1(5), and 3) a possible active role of plasmin in the "steady state" metabolism of IgG.

MATERIAL AND METHODS

The monoclonal IgG isolates were freshly obtained from 48 randomly encountered human sera with well-delineated paraprotein "spikes" > 1.0 g/dl (see Fig. 1, first row). IgG subclassing was done with subclass-specific radial immunodiffusion plates (The Binding Site, Inc., San Diego, CA 92121). The isolates, prepared chromatographically utilizing DEAE Sephadex A-50 and/or Sepharose-linked Protein G (Pharmacia Biotech Inc.,

0006-291X/94 $5.00

Piscataway, NJ 08855), are precipitated with ammonium sulfate at 40 to 50% saturation, as necessary, and the firmly packed precipitates redissolved in a volume of 50% glycerin-stabilized human plasmin equivalent to the starting volume of serum (the stabilized plasmin, "10 CTA units/ml", was a generous gift from colleague Dr. Alan Johnson). The reaction mixtures are then enclosed within Spectra/Por 1 tubing (6,000 to 8,000 MW cutoff), utilizing flat plastic closures (Spectrum, Houston TX, 77073), and dialyzed overnight against 200 volumes of phosphate-buffered saline (PBS), pH 7.4. The "thin-layer" contact of the flatly enclosed reaction mixtures with ambient PBS quickly lowers the plasmin-inhibiting 50% concentration of glycerin, removes residual ammonium sulfate and, at the same time, rapidly brings the ionicity and pH to near physiologic range. Following overnight dialysis, the reaction mixtures are conveniently reconcentrated to approximately the original starting volume of serum by first applying an excess of dry granulated sucrose to the outside of the tubing for about 1 hour. Next the concentrates are re-equilibrated with PBS for approximately 1 more hour prior to being transferred to stoppered tubes for ongoing incubation at room temperature. Plasmin-mediated cleavage is then monitored by scanning densitometry of agarose-gel electrophoresis patterns obtained at designated intervals.

RESULTS AND DISCUSSION

The two IgG3 isolates (nos. 16 & 44, Fig. 1) showed striking susceptibility to plasmic cleavage (100% cleavage overnight). All 40 of the clearcut IgG1 isolates were also susceptible to plasmic cleavage: 20 to 100%

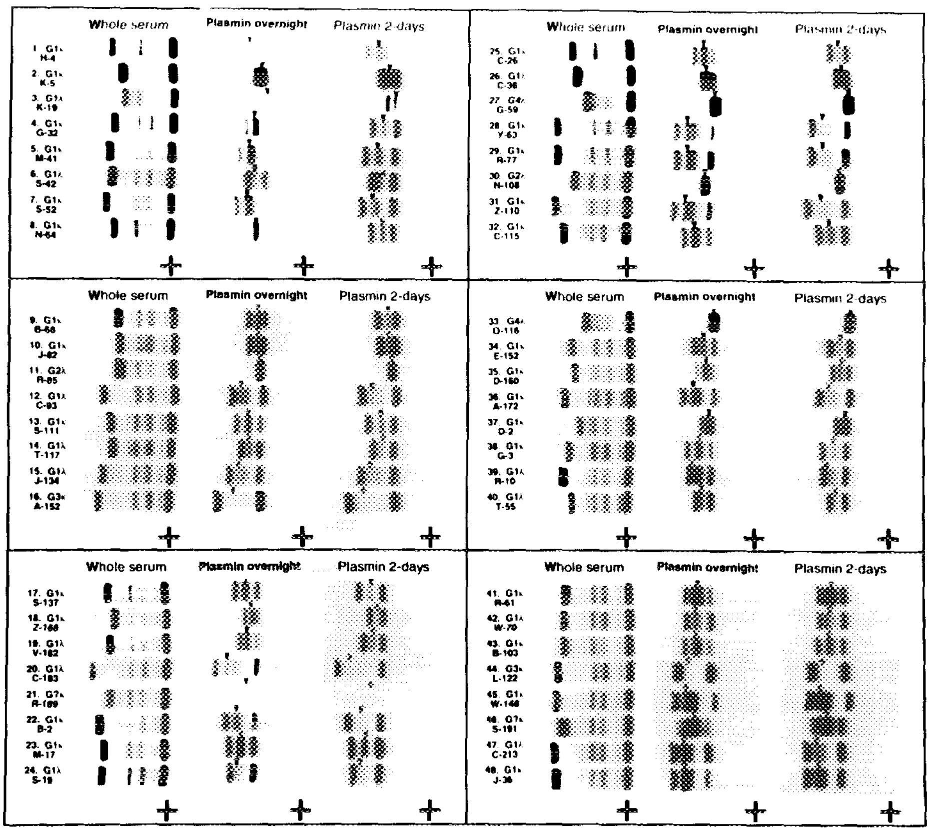

Figure 1. Whole-serum electrophoresis patterns (first row) compared with the plasmic cleavage patterns of the monoclonal IgG isolates following: overnight (middle row), and 2-day incubation at room temperature (last row). The arrowheads in the cleavage patterns indicate the respective positions of the initially intact IgG paraprotein "spikes" prior to plasmic cleavage. The plus signs ("+") indicate the corresponding anodal migration distance of albumin under the electrophoretic conditions employed in the study.

cleavage overnight, 40 to 100% cleavage in 2 days and 60 to 100% cleavage in several days(relative % cleavage was determined as described in the legend for Fig. 3). The two IgG2 (nos. 11 & 30, Figs. 1 & 2) and two IgG4 isolates (nos. 27 & 33, Figs. 1 & 2) were refractory to plasmic cleavage. These findings are consistent with earlier studies conducted by others with both polyclonal IgG (6) and monoclonal IgG isolates (7).

Combined immunoelectrophoresis-electrophoresis, utilizing Paragon SPE plates (Beckman Instruments, Brea, CA 92621), custom-modified (8) for improved resolution and cost efficiency (see legend for Fig. 2), further

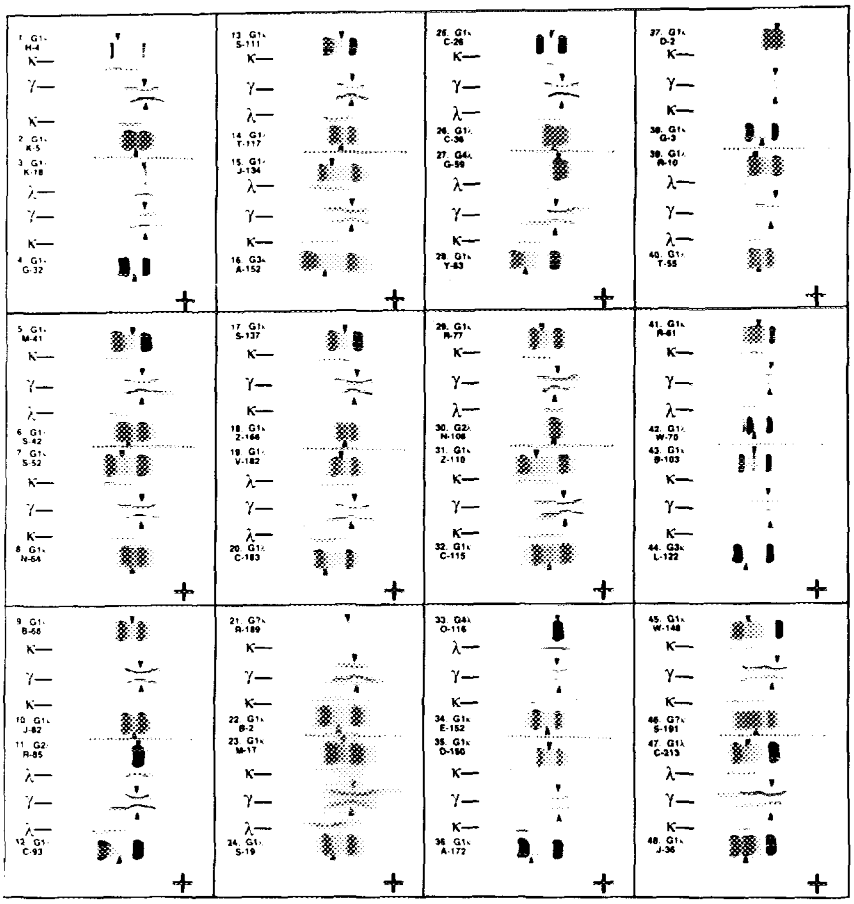

Figure 2. **Combined immunoelectrophoresis-electrophoresis of the 48 monoclonal IgG isolates following several day incubation with plasmin at room temperature.** A 10 μl aliquot of antiserum monospecific for the respective kappa or lambda light chains of each individual paraprotein was applied to each of the sample-track sidewalls exposed by the temporary transfer of the adjacent duplicate sample-tracks for direct protein staining (immediately after electrophoresis). A 20 μl aliquot of Fc-specific anti-IgG was applied to each of the trenches shared in common by each pair of isolates "mirror-imaged" with each other in the respective upper and lower halves of each 76 X 102 mm agarose-gel plate. For the development of the double immunodiffusion patterns the plates were incubated for 2 or 3 hours at room temperature. The arrowheads affixed to the precisely remounted zonal protein reference patterns indicate the respective positions of the intact paraprotein bands prior to plasmic cleavage (cf. Fig 1, first row). The arrowheads above and below each of the shared antiserum trenches (containing Fc-specific anti-IgG) indicate the respective positions of the anodal Fc fragments produced by plasmic cleavage. The plus signs ("+"), as in Fig. 1., indicate the corresponding anodal migration of albumin under the electrophoresis conditions employed.

confirmed that anodal Fc fragments and cathodal Fab fragments are the principal products of the "papain-like" plasmic cleavage of IgG. In addition, in 10 of the IgG1 isolates it revealed transient intermediate banding anodal to the diminishing paraprotein "spikes", but with slower mobility than the Fc fragments, (see: nos. 7,12,20,28,29,31,36,45,47 &48, Figs. 1&2; "Pre-S" bands in nos. 7&45, Fig. 3). Fifteen of the IgG1 isolates also showed continuing plasmic cleavage of the released Fab fragments. This is indicated by the emergence of additional cathodal bands which were nonreactive with Fc-specific anti-IgG and variably reactive with essentially constant-region-specific anti-kappa and anti-lambda (see: nos. 2,4,5,6, 8,9,13,14,19,20,22,41,42,45&46, Figs. 1 & 2; "Fab i" & "Fab ii" bands in nos. 2&45, Fig.3).

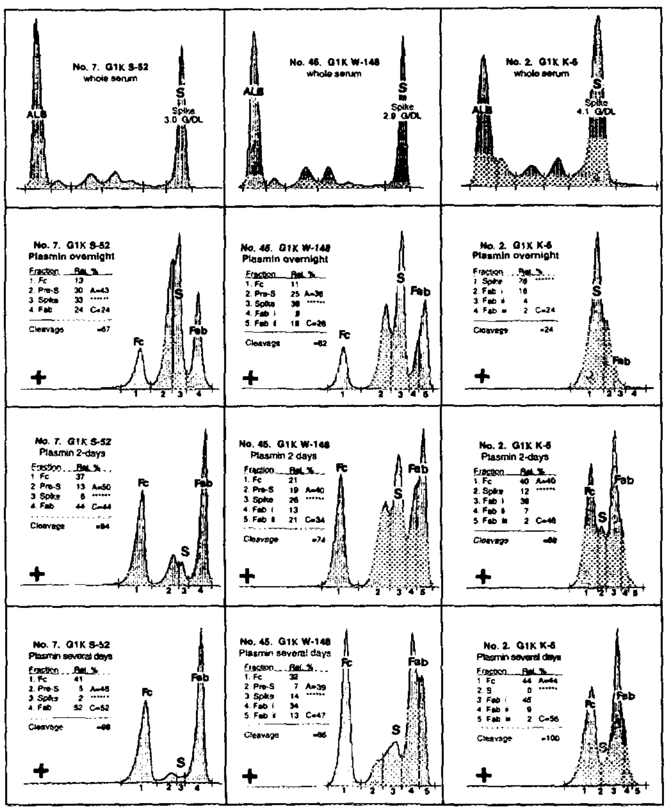

Figure 3. **Representative scanning densitometry of agarose-gel electrophoresis patterns obtained following: overnight, 2-day and several-day incubation of monoclonal IgG isolates with plasmin (see text).** "No. 7. G1K S-52" in the left column illustrates transient banding (designated "Pre-S") anodal to the decreasing paraprotein spike ("S"), but with slower mobility than the progressively increasing Fc band; "No. 45. G1K W-148" in the middle column shows both "Pre-S" banding and two Fab-associated bands ("Fab i" & "Fab ii"), the ratios of which relative to each other are strikingly reversed after several day incubation. "No. 2. G1k K-5" in the right column illustrates Fab-associated multiple cleavage products (designated Fab i, Fab ii, Fab iii) which appeared overnight, prior to the appearance of Fc banding, and increased progressively as the spike (S) progressively decreased. A = sum-total protein anodal to S; C = sum-total protein cathodal to S. The relative % cleavage is calculated as C + A. It should be noted that, following convention, the most anodal components (e.g. albumin and "Fc") are designated "Fraction 1" in the densitometry tracings, which start with the most anodal components to the left, even though in the plates, as actually run in this study, the anodal components migrated to the right (see Figs. 1 & 2).

With respect to the light chain portions of the released Fab fragments, the most likely explanation for further fragmentation is that a series of cleavage-induced configurational changes direct continuing, apparently Fab-fragment-sequestered plasmin activity to the endopeptidase-sensitive junctional region which connects the variable and constant halves of both kappa and lambda light chains (9). This explanation is consistent with the "immunoblot-ligand" studies of IgG cleavage products by Harpel et al. (2), which showed selective binding of plasmin only with plasmin-generated Fab fragments.

Now defined as the "J" segment, the light-chain junctional region is usually comprised of 13 amino acids (e.g. sequence residues 96 through 108), which are encoded for by a germ-line library of J-kappa and J-lambda "minigenes" (10, 11). Interestingly, most of the 40 human, mouse, rat and rabbit J segments so far studied (10) would, by virtue of their high frequency of lysine and arginine residues, appear to be potentially able to contribute to a substrate-associated "array" of "lysine-like" positive charges, which is now believed to be necessary for the initiation of tissue plasminogen activator (tPA)-plasminogen-plasmin interactions (12). It is thus especially of interest that a lysine or arginine at position 103 in 32 of these 40 mammalian J segments appears to be an especially well preserved feature of most immunoglobulin light chains. Moreover, 38 of the 40 J sequences contain at least one lysine or arginine residue, and in 24 of these 38 a lysine residue is present in combination with other lysine or arginine residues, irrespective of the presence or absence of a lysine or arginine residue at position 103 (10).

In the case of lambda light chains, other potential sites for involvement in plasminogen-plasmin interactions would be the antibody-distinguishable lysine ("Oz+") or arginine ("Oz-") residues isotypically preserved at position 193 within the lambda-chain constant domain (13). In the case of IgG1 Fab fragments, other potential sites for involvement in plasminogen-plasmin interactions, depending on the Gm allotype of the Fd (heavy-chain) portions, would be the autoantibody-distinguishable Gm-17-determinant lysine or Gm-4-determinant arginine at position 214 within the gamma-1 first constant domain (14). These Fab-fragment sites for potential involvement in plasminogen-plasmin interactions and the reported IgG1 hinge-region site of plasmic cleavage (1,2) are depicted diagrammatically in Fig. 4.

Studies in progress indicate that the transient anodal bands of intermediate mobility, discussed above, represent the IgG paraprotein molecules from which only one Fab fragment has been initially released by plasmin; in this sense they would be analogous to the "asymmetrical" Y fragments (core E nodules with only one of their two D nodules removed), which have now been shown by both chemical analysis (16) and electron microscopy (12) to be formed transiently during plasmin-mediated fibrinolysis.

Of further interest in this study is the unambiguous demonstration, apparently for the first time, of sequential plasmic cleavage of "released" Fab fragments, the heavy- and light -chain variable domains of which bear the idiotype determinants. Within the physiologic milieu, initial hinge-region cleavage of susceptible immunoglobulins, either by endogenous plasmin or granulocyte elastase(17), would thus be very likely to facilitate further Fab fragmentation leading not only to the release and further processing of any Fab-bound exogenous antigens, but also to further "processing" of the respective heavy- and light-chain idiotype determinants leading

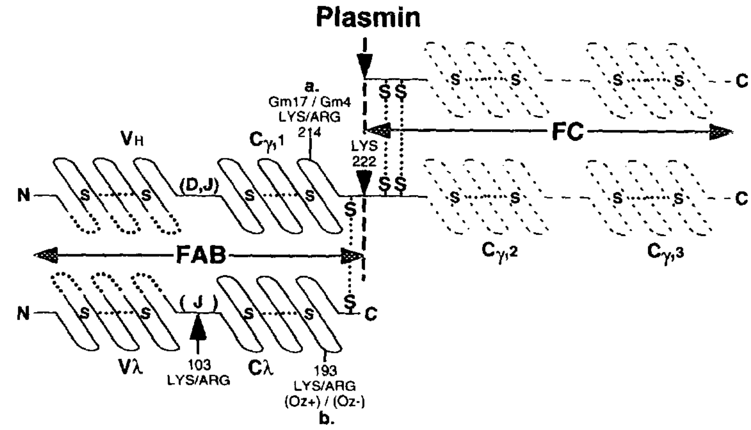

Figure 4. **Some proposed lysine/arginine-mediated plasminogen-plasmin-IgG interaction sites, depicted diagrammatically with a lambda light chain disulfide linked to an intact gamma subclass-1 heavy chain.** The heavy chain is, in turn, disulfide linked to the corresponding Fc segment (upper right) from which the Fab fragment is shown as having already been released by plasmic cleavage (see text). The down-pointing arrowheads indicate the reported plasmic cleavage site on the C-terminal side of the Lys residue at position 222 within the IgG1 hinge region (1,2). The up-pointing arrowhead (lower left) indicates the highly conserved Lys (n= 28) or Arg (n= 4) at position 103 in 32 of the 40 human, mouse, rat and rabbit J-kappa and J-lambda "joiner segments" so far studied (10). Also indicated are: a. the allotypic Gm-17-determinant Lys or Gm-4-determinant Arg proximal to the hinge region at position 214 within the gamma subclass-1 first constant domain (14), and b. the antibody distinguishable Lys ("Oz+") or Arg ("Oz-") isotypically preserved at position 193 within the lambda chain constant domain (13).

...... = Idiotype/complementarity-determining hypervariable regions of both the light and heavy chain components of Fab fragments. ——— = "framework" and constant regions of both the light and heavy chain components of Fab fragments. --------- = continuation beyond the hinge region of the disulfide-linked Fc portions of the IgG1 heavy chains. "S......S" = Interchain disulfide bonds. "s......s" = Intradomain disulfide bonds that "pin" together the "antiparallel beta-pleated sheets", each of which is typically "immunoglobulin-folded" (not depicted in above Fig.) to form two complexly interlocked "layers" respectively comprised of 4 and 3 beta segments (see Fig. 5 in ref. 15). "(D, J)" = The respective "Diversity" and "Joiner" segments that connect the N-terminal variable domain with the 1st constant domain of all immunoglobulin heavy chains (11). "(J)" = the light chain "Joiner" segment, usually comprised of 13 amino acid residues which are encoded for by a germ-line library of J-kappa and J-lambda "minigenes" (10,11).

to the elicitation of immunoregulatory anti-idiotype networks (18). This latter aspect has already been discussed by Bourgois et al. (19) and also by Putnam et al. (20) in connection with the strikingly enzyme-labile hinge region of both mouse and human IgD. Bourgois et al. (19), in fact, proposed that the major role of IgD is "to provide released Fab fragments" that then elicit "anti-idiotype responses which play a crucial controlling role in the genesis of the immune response."

That, under near physiologic conditions, human plasmin can, indeed, readily cleave IgD into Fab and Fc fragments, even at plasmin concentrations too low to produce correspondingly detectable cleavage of IgG, has already been demonstrated by Griffith & Gleich (21). Thus, in addition to "clot busting", finely focalized plasminogen-plasmin activation may also: 1) play a well-programmed ancillary role within the immune system, and 2) play an active role in the essentially steady state metabolism of both circulating and extravascular IgG, which is yet to be adequately explained (22). Taking into account earlier studies by Morgan and Weigle (5), a plausible "steady state" mechanism would be the generation of B-cell-targeted "anabolic" feedback fragments as a direct consequence of the "catabolic" cleavage of IgG1 by plasmin within both the intravascular compartment and interstitial spaces. These and other physiological implications of the apparently well-programmed cleavage of susceptible immunoglobulins by plasmin, as extensively documented with monoclonal IgG1 isolates in this study, clearly warrant further investigation.

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