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4 **Online Repository for IgG Subclass Distributions in Serum**

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15 *Subjects*

16 Subjects were recruited under three Johns Hopkins University IRB-approved
17 protocols for serum collection. Initial collections were quasi-random and atopic
18 history was ascertained solely by historical account of a physician diagnosis of
19 allergies from the subjects, based on skin prick tests and including whether they had
20 obtained some form of allergen immunotherapy. For the subjects who do not report
21 "allergies" in general or to the two allergens examined by ELISA, there was no
22 corroborating skin test data. A secondary group was recruited from the Johns
23 Hopkins Allergy clinic prior to or after they had obtained subcutaneous allergen
24 immunotherapy. Once again, subject atopy was confirmed by skin prick test data.
25 Serum was obtained from blood samples after clotting and stored at -80˚C.

26 *ELISA construction*

27 ELISA plates Nunc MaxiSorp flat bottom plates were coated overnight at room
28 temperature with allergen solutions. Only mixed allergens were used. Lyophilized
29 Short Ragweed (Greer Laboratories) was diluted to a concentration of 4 μg/ml in
30 PBS and 0.3 ml loaded into each well of the plate. The plates were washed 5 times
31 with PBS containing 0.02% Tween-20 and 0.1% BSA before adding samples or
32 standards. Washing after overnight adsorption was followed by 10-15 minutes of
33 blocking in washing buffer containing 0.1% BSA. Dermatophagoides farinae (Df)
34 and Dermatophagoides pteronyssinus (Dp) from Allermed (10,000 BAU/ml stock in
35 50% glycerin) were coated as a mixture, each at 5 BAU/ml final concentration in
36 PBS. Human serum albumin was coated at 2 μg/ml in PBS for the pilot experiments

37 to determine nonspecific binding. Standards and serum samples were incubated in
38 the plates for 1 hour at room temperature in PBS + 0.02% Tween-20 and 0.1% BSA.
39 Typically 1/20 and 1/100 dilutions of the serum samples were added to the assay
40 wells. After 4 washes with PBS/0.02% Tween-20, anti-subclass antibodies (anti-
41 IgG1, HP6070P; anti-IgG2, HP6002P; anti-IgG3, HP6047P; anti-IgG4, HP6023P,
42 diluted in PBS/0.02% Tween-20) were added at concentrations of 10 μg/ml, 4
43 μg/ml, 6 ng/ml and 1 μg/ml, respectively (see next section for calibration choices).
44 After a one-hour incubation at room temperature and 4 washes, a 1/4000 dilution
45 of anti-mouse IgG-HRP (Amersham, Inc.) was added for a one-hour incubation at
46 room temperature. After 5 washes, TMB solution was added and the reaction
47 stopped with 1M H_2SO_4.

48 In pilot experiments, nonspecific binding in this ELISA format (with serum present
49 in the first incubation) was assessed by comparing binding in wells coated with HSA
50 or coated with ragweed. For the IgG1, 2 and 4 subclasses, nonspecific binding to HSA
51 was less than 1-5% and about 7% for IgG3. Sera were also adsorbed with sepharose
52 beads coupled to either HSA or ragweed (CNBr-enabled coupling) and retested in
53 the ELISA; approximately 90% of the signal was lost by adsorption with ragweed-
54 beads and not with HSA-beads. In addition, dilution studies of test sera
55 demonstrated that there was not an indication that one antibody subclass could
56 block access by other subclasses, notably, that antigen-specific IgG1 was not
57 overwhelming the ability of lower concentrations of IgG3 or IgG4 to be detected.

58 *Cross-calibration of anti-subclass antibodies*

59 The transfectoma series of subclass antibodies (IgG1, IgG2, IgG3, IgG4) [1] specific
60 for a p-nitrophenyl ligand were used to cross-calibrate the anti-subclass antibodies.
61 Pilot studies calibrated these transfectoma antibodies with a total IgG ELISA where
62 the detection antibody was the WHO Ab 6045P [2]. Human IgG (ICN Laboratories)
63 was used as the standard. With equal concentrations of the transfectoma subclass
64 antibodies incubated on a plate, the anti-subclass antibodies were titrated to
65 generate similar optical densities in the final reading. This balancing resulted in the
66 concentrations of the anti-subclass antibodies used above (10 μg/ml, 4 μg/ml, 6
67 ng/ml, and 1 μg/ml for anti-IgG1, HP6070P; anti-IgG2, HP6002P; anti-IgG3,
68 HP6047P; anti-IgG4, HP6023P, respectively).

69 For absolute calibration, on a single plate, one-third was loaded with ragweed (4
70 μg/ml in PBS), a third loaded with NP(7)-BSA (10 μg/ml in PBS) and a third loaded
71 with equal mixture of D.P and D.F. dust mite allergens described above. For the
72 ragweed portion, the serum standard was loaded at a range of concentrations while
73 for the portion adsorbed to NP-BSA, anti-NP specific IgG1, IgG2, IgG3 or IgG4 was
74 loaded at 15 ng/ml (for each subclass) and in the portion adsorbed to dust-mite, the
75 chosen serum standard for dust-mite assays. After a one-hour incubation and
76 washing, anti-subclass antibodies (see above) were incubated for 1 hour at room
77 temperature. After washing, the wells were incubated with anti-mouse HRP
78 antibody for 1 one-hour at room temperature. After washing, TMB solution was
79 added and the color reaction stopped with 1M H_2SO_4. From the titration curves for a
80 serum standard and subclass antibodies, equivalence (equal optical density) was
81 calculated for the serum standard. These results allowed the absolute

82 concentrations of anti-ragweed subclass antibody to be determined and cross-
83 calibrated the ragweed and dust mite standards for each subclass.

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85 **References**

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87 functions of human immunoglobulins using a matched set of chimeric
88 antibodies. Journal of Experimental Medicine 166, 1351-61.
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