Direct immunofluorescence (IF) is used in the detection of autoantibodies. Positivity is recognized as bright green fluorescence at the site of antibody-antigen interaction (1). Normal human serum gives the appearance of dull, blue-green or yellow-green staining depending upon the use of a counterstain and the selected barrier filter on the fluorescence microscope. This normal background fluorescence results from the nonspecific binding of the secondary fluorochrome-conjugated antibody. A failure to add either secondary antibody or serum during testing results in a noticeable absence of nonspecific fluorescence.

During examination of rodent liver-kidney-stomach (LKS) sections, occasional samples (<0.5%) demonstrated this markedly reduced level of overall fluorescence. In some cases, the LKS sections appeared dull red, suggesting that a failure to add either the serum or the secondary antibody had occurred. However, the observation was reproducible and was also noted during testing for antidendymal antibodies (EMA) with monkey esophagus (MO). This appearance is referred to as “dark IF” throughout this report. Dark IF was less apparent on HEp-2 cells, possibly due to the higher screening dilution of the test samples, reducing the level of overall nonspecific fluorescence; therefore, this report describes results with LKS and MO only.

Local Research Ethics Committee (North and Mid Essex, United Kingdom) approval was obtained to explore the hypothesis that abnormalities in serum immunoglobulins (Igs) may be responsible for dark IF, a theory prompted by our observation of this phenomenon in a patient with known hypogammaglobulinemia. Since this initial observation, it has been standard practice to note the presence of dark IF when present. Retrospective data could therefore be collected for all samples recorded independently by two experienced screeners to have dark IF from June 2001 to December 2002. IF screening was performed without knowledge of the serum Igs; these data were subsequently available for 49 samples (1 sample was later discarded due to incomplete data).

For the selection of a representative control group (five times the size of the dark IF group), all requests for autoantibody testing and serum Ig analysis received by the laboratory during this 18-month period were considered (9,500 autoantibody test requests; 4,100 serum Ig analyses). To be eligible for inclusion in the control group, subjects had to have autoantibody screens not demonstrating dark IF and serum Ig results during this 18-month period were considered (9,500 autoantibody test requests; 4,100 serum Ig analyses). To be eligible for inclusion in the control group, subjects had to have autoantibody screens not demonstrating dark IF and serum Ig results (n = 357). The first 245 cases (ordered alphabetically) were selected. Data analyzed included age, sex, findings for indirect immunofluorescence, serum Igs, and serum protein electrophoresis (SPE). The male/female ratios were similar in the two groups, 0.6:1 (dark IF) and 0.5:1 (control), as were the mean ages (standard deviations [SD]), 46.1 years (26.9) in the dark IF group and 52.1 years (23.4) in the control group.

Serum tested on LKS substrate was diluted 1/20 in phosphate-buffered saline (PBS). PBS, LKS substrate, and fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (heavy and light chains), containing Evan’s Blue counterstain as the second-stage antibody, were from BioDiagnostics Ltd. (United Kingdom). Slides were viewed at a magnification of ×100.

EMA screens were performed by using MO slides with FITC-conjugated anti-human IgA, containing Evan’s Blue counterstain as the second-stage antibody. Serum was diluted 1/5 in PBS. All reagents were manufactured by Biosystems (Spain) and supplied by Launch Diagnostics (United Kingdom). Slides were viewed at a magnification of ×100.

Serum Ig levels were determined by immunoturbidimetry with an Olympus AU640 (Olympus, Hamburg, Germany) instrument. SPE was performed with the Sebia Hydrasys agarose gel electrophoretic system (Sebia, Atlanta, Ga.).

Serum Igs were considered abnormal if any Ig isotype (IgG, IgA, or IgM) was reduced or if a paraprotein was present. For adult subjects (age, 16 years or greater), reduced Ig levels were defined as follows: IgG, <5.4 g/liter; IgA, <0.8 g/liter; IgM, <0.5 g/liter. For pediatric subjects, appropriate age-related reference ranges were applied (2).

The most notable finding was the more-than-threefold-higher incidence of serum Ig abnormalities in the dark IF group (70.8%) than in the control group (21.6%). Upon evaluation of the mean number of abnormal indices per subject,
this was demonstrated to be statistically highly significant, with
the control group showing 1.32 (SD, 1.15) abnormalities per
subject compared with 0.29 (SD, 0.64) in the dark IF group
\((P \leq 0.0001)\).

Data from MO and LKS screenings were then analyzed
separately (Table 1). For MO, 83.3% had abnormalities in
serum Igs in the dark IF group, compared with 27.8% of
controls. While the most common abnormality was an isolated
decrease in IgA, all subjects with abnormal findings in the dark
IF group had reduced IgA levels. This is in keeping with the
IgA-specific second-stage antibody used on MO sections.

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