Interleukin-10 concentration determined by sandwich enzyme-linked immunosorbent assay is unrepresentative of bioactivity in murine blood

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Hillyer, L. M. and Bill Woodward. Interleukin-10 concentration determined by sandwich enzyme-linked immunosorbent assay is unrepresentative of bioactivity in murine blood. Am J Physiol Regul Integr Comp Physiol 285: R1514–R1519, 2003; 10.1152/ajpregu.00378.2003.—Two experiments were performed, each using six male and six female C57BL/6J mice collectively ranging from 4 wk to 17 mo of age. Blood was obtained following CO₂ anesthesia, and the IL-10 concentration of each serum sample was determined both by sandwich enzyme-linked immunosorbent assay (ELISA) and by bioassay. In the first experiment, mean serum IL-10 immunoactivity was 9.3 pg/ml while the mean bioactivity was 700 times greater, i.e., 6.5 ng/ml. However, the bioassay required sample dilution, which might have released bound cytokine that the ELISA could also detect. In the second experiment, therefore, the ELISA was applied to samples diluted to 20% as for the bioassay. Nevertheless, the immunoassay continued to detect only a small fraction of the serum IL-10 identified by the bioassay (mean values: 32.4 pg/ml vs. 2.6 ng/ml). Although currently the preferred method, the sandwich ELISA is inappropriate for quantification of blood IL-10 concentrations. Moreover, studies of the actions of IL-10 are needed at the concentrations revealed in the blood by bioassay and currently considered supraphysiologi cal.

bioassay; cytokine; immunoactivity; mouse

Interleukin-10 potently inhibits cell-mediated inflammation (21). This is accomplished primarily by downregulating the expression of inflammatory cytokines, an influence mediated through mononuclear phagocytes and other accessory cells (21), but is also effected by inhibiting the actions of some inflammatory cytokines, notably IL-1 and tumor necrosis factor (21). The anti-inflammatory action of IL-10 appears critical to physiological functions such as self-tolerance (21) and the maintenance of pregnancy (5). In terms of infectious disease resistance, IL-10 is regarded as central to the balance that must be established between immunological protection and accompanying inflammatory damage (21).

IL-10 is an autocrine and paracrine hormone (21), but its blood concentrations are of interest for a variety of reasons. For example, the therapeutic application of IL-10, e.g., as an anti-inflammatory agent in the management of infections in humans and animals (15), renders monitoring blood levels a clinical imperative. The blood IL-10 concentration also may prove to be a valuable marker in the management of numerous malignancies (1, 28, 30) and diverse inflammatory conditions (9, 11, 13, 29, 35, 40, 42), including cardiovascular disease (12, 20) and tissue transplant reactions (36) as well as in the diagnosis and treatment of hypercortisolemic conditions (8). In addition, blood levels of cytokines including IL-10 are of interest for research into the immunological characteristics of diverse pathologies (6, 25–27, 33, 38, 39, 43) as well as of physiological conditions such as pregnancy (5), advancing age (24), and the response to exercise (37). Blood concentrations of cytokines may prove particularly informative when these regulatory molecules are assessed in panels to reflect “balance at the systemic level” (38).

The sandwich enzyme-linked immunosorbent assay (sandwich ELISA) is the most popular technique for estimation of cytokine concentrations in biological fluids, including the blood (4, 19). This is primarily because of the cost-effective speed and simplicity of the assay combined with its specificity. However, cytokines in the blood are predominantly bound to numerous other proteins, e.g., receptor fragments, autoantibodies, and α2-macroglobulin, and the sandwich ELISA is widely considered to detect only the unbound fraction (4, 18). Consistent with this model, the sandwich ELISA was recently reported to detect only 2% of the IL-1, IL-6, and IL-10 actually present in the blood according to a competitive binding assay (18). Likewise, blood cytokine concentrations determined by sandwich ELISA are generally presumed unrepresentative of biological activities because of the inability of an ELISA to discriminate between biologically active and inactive molecules bearing the epitope(s) that the assay is designed to detect (4). This is a reasonable and significant proposition but is in need of experimental testing. Therefore, the objective of the present investigation was to compare the blood IL-10 concentrations...
detected by a widely used sandwich ELISA and a recognized bioassay.

MATERIALS AND METHODS

Animals and animal facility. Male and female C57BL/6J mice were used from an in-house breeding colony derived from animals originally purchased from the Jackson Laboratory (Bar Harbor, ME). Caging and other environmental conditions were as described previously (16, 34), and animals had continuous free access to clean tap water and rodent chow (Rodent Laboratory Chow 5001,Ralston Purina International). According to serologic analysis of sentinel mice, the colony is free of common viral pathogens (Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse polyoma virus, reovirus type 3, and mouse parvovirus) and Mycoplasma pulmonis. The investigation was conducted in accordance with the guidelines of the Canadian Council On Animal Care and was approved by the Animal Care Committee of the University of Guelph.

Experimental design. Two independent experiments were performed. Three age groups of mice, i.e., 4 wk, 4 mo, and 17 mo postpartum, were included in the first experiment to represent postweaning life from its beginning into advanced age. Four mice, two males and two females, were included in each age group for a total of 12 animals from six litters. The second experiment included two age groups of mice, i.e., 4 wk and 9 mo of age, and three males and three females were included in each age group to yield a sample size of 12 animals from eight litters. In both experiments, sufficient blood was taken from each mouse to permit measurement of the IL-10 concentration by both sandwich ELISA and bioassay.

Blood collection and sample preparation. Orbital plexus blood was taken from mice under CO2 anesthesia as described previously in this laboratory (34), after which the blood was allowed to clot at room temperature described previously in this laboratory (34), after which the serum was removed from each well for assay of interferon-γ concentration by ELISA. A commercial ELISA kit was used for this purpose (OptEIA kit, BD Biosciences) but without preparation of a standard curve because transformation of absorbance readings into interferon-γ concentrations was unnecessary. In our hands, this ELISA typically exhibits a detection limit not exceeding 5 μg/ml, an intra-assay coefficient of variation of 3–5%, and a standard curve conforming to linearity, i.e., R2 ≥ 0.98, over the full range of the reliably detectable absorbance readings of a Vmax kinetic plate reader (unpublished results).

An intra-assay coefficient of variation was calculated for the bioassay by determining the mean and SD of outcomes from four culture wells containing recombinant IL-10 at a concentration in the midrange of the linear portion of the standard curve. Moreover, because the usable portion of the standard curve was confined to its linear portion, the limit of detection for the bioassay was taken as the well IL-10 concentration at the lower limit of the linear region of the curve. Finally, in preliminary studies, the specificity of the bioassay for IL-10 was demonstrated by inclusion of 4 μg of anti-murine IL-10 (clone: JESS–2A5, rat IgG1) in wells containing mouse serum (n = 4) during the 24-h incubation stage of the assay, i.e., to achieve an antibody concentration of 40 μg/ml in the cultures. The antibody eliminated detectable inhibition of interferon-γ production by each of the four serum samples used in this test.

Statistical analysis. Statistical analyses were performed using the SAS system for windows (31) and applying a predetermined upper limit of probability of P = 0.05 for a decision of statistical significance. In each experiment, the dataset produced by combining the outcomes of the ELISA and the bioassay resisted transformation to normality. Consequently, in the first experiment, comparison of the outcomes of the two assay procedures was achieved by means of a paired sign test, i.e., pairing the estimates of immunoactivity and bioactivity within each serum sample, using the χ2 statistic. Comparison of the three assays of the second experiment was carried out by means of the Kruskal–Wallis test (χ2 approximation) applied to Wilcoxon rank sums. As warranted by the resulting statistical probability value (P ≤ 0.05), the procedure was followed by χ2 comparisons of Wilcoxon two-sample rank sums. In addition, Pearson correlation analysis was used to search for an association between...
the outcomes of the sandwich ELISA and the bioassay in each experiment.

RESULTS

Experiment 1: comparison of sandwich ELISA and bioassay, each conducted under optimized conditions. The standard curve established for the IL-10 ELISA is shown in Fig. 1 together with a measure of its linearity ($R^2 = 0.9941$) and estimates of both the reliability (coefficient of variation = 1.3%) and the detection limit (0.4 pg/ml) of the assay. The standard curve for the bioassay conformed to an exponential function over the range of in-well concentrations tested, i.e., 0.14–9.0 ng/ml produced by serial doubling dilutions (not shown). Figure 2 shows the usable linear portion of the curve (extending over the in-well concentration range of 0.56–2.25 ng/ml) together with an assessment of the linearity of this region ($R^2 = 0.9985$), the coefficient of variation of the assay (3.1%), and its limit of detection (0.6 ng/ml). Because all standards and sera were diluted to 20% for the bioassay, the cited detection limit corresponded to a sample concentration of 3 ng/ml. All serum samples yielded readings within the linear portion of the standard curve of both the ELISA and the bioassay.

Paired comparison of serum IL-10 immunoactivities and bioactivities (Fig. 3) revealed that the mean IL-10 concentration assessed by bioassay (6.5 ng/ml) exceeded the mean immunoactivity determined by sandwich ELISA (9.3 pg/ml) by a factor of 700 times ($P < 0.005$). Moreover, correlation analysis revealed no association between the bioactivity and immunoactivity outcomes ($r = -0.10, P = 0.77$).

Experiment 2: comparison of sandwich ELISA and bioassay controlling for serum dilution. The results of the first experiment were obtained using a bioassay in which serum samples were diluted to 20% and an ELISA in which undiluted serum samples were assessed. It was possible that the high IL-10 levels revealed by bioassay reflected a dilution-related shift in equilibrium between bound and free cytokine that the ELISA would also detect if applied to samples diluted similarly. Hence, IL-10 concentrations of serum samples, diluted to 20% in complete medium, were determined by both sandwich ELISA and bioassay. As a positive control, undiluted samples were also subjected to sandwich ELISA as in the first experiment.

The limit of detection, intra-assay coefficient of variation, and standard curve linearity for the ELISAs were 3.6 pg/ml, 5.6%, and 0.9996, respectively. Assessment of the same quality control indexes in relation to the bioassay yielded values of 0.6 ng/ml, 4.8%, and 0.9545. Figure 4 presents the results from the bioassay of the 12 serum samples together with the outcomes from the assay of the same samples, both undiluted and diluted to 20%, by sandwich ELISA. All serum samples yielded optical density readings within the linear portions of both standard curves. The diluted serum samples yielded a higher estimate of serum IL-10 concentration.
IL-10 immunoactivity than the undiluted samples (mean values: 32.4 vs. 3.9 pg/ml; \( P = 0.05 \)). Nevertheless, the bioassay yielded an estimate of mean serum IL-10 concentration (2.6 ng/ml) that exceeded, by a factor of \( \sim 80 \) times, the estimate of immunoactivity resulting from application of the ELISA to the same serum dilution (\( P < 0.0001 \)). No correlation was found between the bioactivities and immunoactivities assessed to the diluted serum samples (\( r = 0.31, P = 0.33 \)). Likewise, in confirmation of the results of the first experiment, no association was apparent between IL-10 bioactivity and IL-10 immunoactivity when the latter was determined using undiluted serum samples (\( r = 0.35, P = 0.26 \)).

**DISCUSSION**

This investigation provides direct evidence that IL-10 immunoactivity determined by sandwich ELISA in the blood serum of the mouse provides no insight into the bioactivity of IL-10 in this biological fluid. This conclusion is based both on the quantitative difference between the outcomes of the two types of assay and on the lack of correlation between them. In this regard, it should be noted that this investigation covered post-weaning life into advanced age and that both sexes were equally represented. Moreover, the difference between the IL-10 concentrations detected by ELISA and by bioassay clearly cannot be attributed either to the inter- and intra-assay variation associated with the techniques (this investigation) or to the variation associated with personnel (unpublished comparison among 3 users in this laboratory, 3-fold range in means, each method). The serum IL-10 immunoactivity detected by the sandwich ELISA used in this investigation varies inversely with the serum concentration to which the capture antibody is exposed (results not shown, concentrations ranging from 1.6 to 100%, \( n = 4 \)). Presumably this reflects a progressive shift of the equilibrium position toward unbound cytokine as serum dilution increases. Nevertheless, when serum dilution was eliminated as a variable between the bioassay and the sandwich ELISA, the ELISA detected little more than 1% of the IL-10 concentration revealed by the bioassay. Previously, the sandwich ELISA was reported to detect only 2% of the IL-10 immunoactivity identified in human serum by a competitive binding assay (18). The sandwich ELISA, therefore, is inappropriate for the assessment of blood IL-10 concentrations as this assay misrepresents both the quantity of IL-10 protein in the blood (18) and the blood IL-10 bioactivity (this investigation). More broadly, the results of the present investigation constitute a proof-of-concept probably relevant to the assay of blood concentrations of many cytokines, and applicable across species.

The bioactivity of IL-10 in the blood does not appear to have been reported previously. Taken together, the two experiments of this investigation define the normal serum IL-10 bioactivity of the C57BL/6J mouse to be 5 ± 3 ng/ml (mean ± SD) and show that this characteristic is independent of age and sex (\( P \geq 0.18 \) and \( P \geq 0.32 \), respectively, 2-way ANOVA, results not shown). Previous reports of the blood IL-10 concentration in the laboratory mouse (2, 7, 26) are based on application of the sandwich ELISA to the blood of young adult animals, and the reported range of normal concentrations extends from <4 pg/ml (26) to as much as 150 pg/ml (2). The present results pertaining to blood IL-10 immunoactivity are consistent with this database and extend it to include the life span of the mouse from weaning to advanced age. However, despite an apparently broad range of normal murine blood IL-10 immunoactivities, the highest blood IL-10 immunoactivity reported to date for the healthy mouse (2) is only \( \sim 3 \% \) of the bioactivity detected in the present investigation. Similarly, the sandwich ELISA reveals a broad range of blood IL-10 immunoactivities, albeit confined to picogram per milliliter levels, throughout the life span of overtly disease-free humans (8, 9, 11, 13, 20, 22, 24, 27, 29, 30, 32, 33, 36–40, 43), and concentrations ranging from 1–2 pg/ml (9, 24, 40), or less (11, 39) to levels as high as 70 pg/ml (43) and even 400 pg/ml (29) are reported. Different ELISA reagent combinations commonly yield cytokine analyses that vary by more than an order of magnitude (4, 14, 19), and this may contribute to the wide range of normal blood IL-10 immunoactivities reported in humans and animals. The common perception that cytokine immunoassays are more accurate and reliable than bioassays is misplaced (19), and the bioassay, despite its inconvenience and specificity problems (4), deserves renewed attention as the gold standard at least where blood cytokine assay is concerned. Cytokines including IL-10 are also of interest in other biological fluids, e.g., synovial fluid (3), peritoneal washings (25), subretinal fluid (17), and subepidermal blister fluid (32). On the basis of the present investigation, it is reasonable to conclude that the suitability of the sandwich ELISA requires verifi-
cation for the assessment of cytokine concentrations in any biological fluid.

Cytokines are widely considered to exert their influences, in vivo, at concentrations within the picogram per milliliter range (10). This point of view is fueled by two observations. In the first place, cytokines are biologically potent in vitro at picogram per milliliter concentrations (4, 10). Second, apart from pathological conditions, the concentrations reported in biological fluids, particularly in the blood, are almost uniformly in this range (4). Although it is recognized that the blood is unlikely to provide an accurate representation of local extravascular cytokine levels (4), blood immunooactivities determined by sandwich ELISA are frequently used as a guideline to define “physiological” cytokine levels (e.g., 41). In effect, therefore, blood cytokine concentrations assessed by sandwich ELISA are an integral part of the foundation on which current understanding of cytokines is based. Reappraisal of the biological actions of IL-10 is warranted through studies of concentrations comparable to the blood bioactivity identified in this investigation, and a need for similar reassessment is probable in relation to many other cytokines.

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DISCLOSURES
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REFERENCES


