Reduced Primary Antibody Responses in a Genetic Animal Model of Depression

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Objective: Clinical depression is associated with multiple abnormalities of immune function, including reduced virus-specific responses. This study tested the hypothesis that the Flinders Sensitive Line (FSL) rat, a promising genetic animal model of depression, would exhibit reductions in antigen-specific primary antibody responses to immunization. Methods: FSL (N = 13) and control Flinders Resistant Line (FRL; N = 14) rats were immunized with the protein antigen keyhole limpet hemocyanin (KLH; 300 μg/kg), and KLH-specific immunoglobulin (IgM, IgG, IgG1, and IgG2a responses were measured before and 3, 5, 7, 11, and 14 days after immunization. In separate experiments, production of interferon-γ (IFN-γ) by cells from naive and KLH-immunized animals was measured in vitro to determine whether strain differences in antibody production might be associated with differential production of this regulatory cytokine. Results: KLH-specific production of IgM (p < .01) and IgG2a (p < .05) was significantly reduced in the FSL rats compared with the FRL controls. There were no strain differences in IgG or IgG1 production. Although IFN-γ production between the two strains was similar in naive animals, cells from KLH-immunized FSL rats produced significantly less IFN-γ when stimulated with KLH in vitro than cells from KLH-immunized FRL controls (p = .01). Conclusions: This study extends previous reports of altered immune function in the FSL rats to include reduced in vivo antigen-specific antibody responses. Moreover, diminished production of IFN-γ by KLH-primed lymphocytes may contribute to lower antibody production in these animals. Collectively, these data suggest deficiencies in type 1 T-helper cell-mediated immunity in the FSL rats. Key words: depression, Flinders, antibody, keyhole limpet hemocyanin, interferon-γ.

INTRODUCTION

Major depressive illness is reliably associated with abnormal immune function. Compared with matched nondepressed control subjects, for example, individuals diagnosed with clinical depression exhibit significant decreases in natural killer (NK) cell cytotoxicity (1–5) and lymphocyte proliferative responses (6, 7), altered cytokine production by stimulated lymphocytes in vitro (8, 9), reduced delayed-type hypersensitivity responses in vivo (10), increased plasma concentrations of some cytokines and soluble cytokine receptors (11–13), and marked reductions in virus-specific immunity (14). We and others have previously reported depression-like abnormalities of immune function in Flinders Sensitive Line (FSL) rats, a promising genetic animal model of depression. The aim of the current research was to test the hypothesis that antigen-specific in vivo immune responses would also be reduced in the FSL model.

The FSL rats, and their control counterpart, Flinders Resistant Line (FRL) rats, were originally developed by selectively breeding Sprague-Dawley rats for greater (FSL) or lesser (FRL) sensitivity to the hypothermic effects of the cholinesterase inhibitor diisopropylfluorophosphate (15), and subsequent generations of these two strains have maintained their differential sensitivities (16). Additional studies have shown that compared with the FRL strain, the FSL rats exhibit a number of physiological and behavioral abnormalities that are consistent with clinical depression, including greater muscarinic receptor sensitivity (17), increased rapid eye movement sleep with a decreased latency to the first rapid eye movement episode (18), a shorter circadian period during free run (19), increased behavioral immobility during forced swimming (20), and delayed acquisition of tasks involving active avoidance (21). Immunological analyses of the Flinders animals have shown that the FSL animals exhibit greater susceptibility to anaphylaxis after ovalbumin administration (22) and reduced NK activity per cell under resting conditions (23) compared with the FRL strain. Because declines in NK cytotoxicity and increased atopy are both observed in clinical depression, these data support the potential value of the FSL model for understanding depression-related immune dysfunction. Other aspects of immune function and inflamma-
I. INTRODUCTION

The present research was designed to extend our understanding of the immunological responses in FSL rats, a model strain of rats that exhibit altered stress responsiveness compared to the FRL control strain. The FSL rats are characterized by increased corticosterone levels compared to FRL controls, which may be due to differences in the production of interferon-γ (IFN-γ) and other proinflammatory cytokines. The goal of this study was to investigate the immunological differences between FSL and FRL rats after exposure to psychological stress.

II. MATERIALS AND METHODS

A. Animals

The subjects for this study were male FSL (N = 13) and FRL (N = 14) rats bred at the University of North Carolina, Chapel Hill. After shipment, all rats were housed in pairs in an isolated animal room at Williams College and given at least 2 weeks to adjust to the new environment. The housing environment was maintained at 22°C with lights on at 0600 and off at 1800. Food and water were available ad libitum. To prevent exposure to pathogens, rats were housed in Plexiglas cages with specialized Micro-Isolator filters affixed to the cage lids (Lab Products, Inc., Maywood, NJ). All rats were handled for 2 weeks before experimental procedures to reduce the stress associated with human contact and to habituate the rats to the restraint device used for blood sampling. The experimenters remained blind to the strain of each animal until the conclusion of all experiments.

B. Immunization and Blood Sampling

All rats were immunized intraperitoneally (IP) with the soluble protein antigen KLH (300 μg/kg KLH ammonium sulfate slurry; Calbiochem, La Jolla, CA) in sterile phosphate-buffered saline (PBS; Sigma Chemical Co., St. Louis, MO). Previous research in our laboratory has shown that a 300-μg/kg dose of KLH induces robust antibody responses (30, 31). Blood samples for titration of antibody were obtained 1 day before the experimental manipulation (day 0) and again on days 3, 5, 7, 11, and 14 after immunization using a modification of a simple tail vein puncture method for bleeding unanesthetized rats (34). This method does not produce physical trauma, and repeated blood sampling of up to 1 ml does not alter corticosterone levels compared with levels found in naive animals (34). Briefly, rats were restrained, the tail was briefly immersed in warm water, and a 23-gauge needle was inserted into a vessel in the midventral surface of the tail. Blood (approximately 0.3 ml) was collected using heparinized microcapillary tubes and spun in a hematocrit centrifuge for 15 minutes to obtain serum. Aliquots of serum were diluted 1:10 with 0.05% Tween-20 in PBS and stored at −80°C for subsequent analysis by enzyme-linked immunosorbent assay (ELISA).

C. Determination of IgM, IgG, IgG1, and IgG2a antibody titers.

Serum samples were thawed; diluted (1:10, 1:25, 1:75, and 1:225) in IgM plates; 1:50, 1:250, and 1:500 in IgG plates; 1:50, 1:250, and 1:500 in IgG1 plates; and 1:50, 1:250, and 1:500 in IgG2a plates. Titration was calculated from positive control serum run with each assay.

D. Statistical Analyses

Inter- and intraassay coefficients of variation for the antibody ELISAs were calculated from positive control serum run with each plate and were typically <0.05 and always <0.10. Data from each experiment were analyzed in two-way mixed analyses of variance.

1 150 mg 2,2′-Azino-bis-(3-ethylthiazoline-6-sulfonic acid) (Sigma) plus 500 ml of 0.1 M anhydrous citric acid (Fisher Scientific, Pittsburgh, PA) in ddH₂O at pH = 4.35.
with repeated measures. The within-subjects factor was day of blood sample (six samples: days 0, 3, 5, 7, 11, and 14), and the between-subjects factor was rat strain (FSL or FRL). Significant main or interaction effects were tested post hoc using a Tukey test. Effects were significant at the p < .05 level.

RESULTS: STUDY 1

Immunization with 300 μg/kg KLH induced robust increases in anti-KLH IgM and IgG antibodies in all animals over the course of the 14 days (p < .0001). Consistent with our hypotheses, serum IgM levels were significantly lower in the FSL rats than in the FRL controls every day blood was drawn after immunization (p < .01; Figure 1, A). In contrast, there were no strain differences in IgG levels at any of these times (p = .45; Figure 1, B). We hypothesized that strain differences in IgG may be confined to one of the subtypes of IgG and that measurement of IgG alone may have masked this effect. Inescapable shock, for example, suppresses production of IgG2a but not IgG1 (35). To test this hypothesis, we measured anti-KLH IgG1 and IgG2a levels in the same samples used for IgM and IgG analyses. The data supported our hypotheses. Although KLH induced robust increases in both IgG subtypes (p < .0001), IgG2a titers were significantly lower in the FSL rats than in the FRL rats (p < .05), particularly on days 11 and 14 after immunization (p < .001), whereas IgG1 levels did not distinguish the two strains (p = .77; Figure 2).

In agreement with observations from previous studies (21, 23), the FSL rats (424 g) weighed significantly less than the FRL controls (511 g) at the beginning of the study (f(25) = 4.05, p < .001). Regression analyses were performed to examine the relationship between weight and antibody responses, and no relationships were observed within strain.

MATERIALS AND METHODS: STUDY 2

Rationale

The results of the first study showed selective reductions in IgG2a in the FSL rats. Because IgG2a production is stimulated by Th1 cytokines and IgG1 production by Th2 cytokines, we hypothesized that diminished IFN-γ production by FSL Th1 cells may contribute to reduced IgG2a responses; we previously reported no strain differences in production of interleukin-2 (IL-2), the other major Th1 regulatory cytokine (23), although these earlier data were from unimmunized animals. We tested this hypothesis by stimulating splenocytes from both naive and KLH-immunized animals in vitro and measuring cytokine release. Simulation of naive splenocytes was expected to reveal nonspecific strain differences in cytokine production, if any, like those observed in stressed animals (35). In contrast, stimulation of KLH-primed splenocytes tested the hypothesis that antigen-specific cytokine production would be lower in the FSL rats than in FRL controls.

Experiment 1: Nonspecific IFN-γ Production

Animals. FSL (N = 8) and FRL (N = 8) rats bred at the University of North Carolina, Chapel Hill, were housed in pairs in an isolated animal room at Williams College and given at least 2 weeks to adjust to the new environment. Housing conditions were the same as those in Study 1. The experimenters remained blind to the strain of each animal until the conclusion of all immunological procedures.

Cell preparation for cytokine analyses. Rats were rapidly decapitated, and spleens were removed to culture plates containing sterile PBS and teased apart with sterile 18-gauge needles. Splenocytes were aspirated, underlayered by Ficoll-Hypaque (Sigma), and spun for 30 minutes at 1200 rpm. The mononuclear cell (MNC) fraction was removed, washed twice with PBS, tested for viability by trypan blue exclusion, and resuspended in complete medium: 1:1 RPMI
Simulated production of IFN-γ was determined using a commercial cytokine ELISA kit (Genzyme, Cambridge, MA) following the manufacturer’s protocol. Standards and samples were plated in duplicate, and plates were read at 450 nm in a spectrophotometer (Molecular Devices). Data are expressed as picograms per milliliter.

**Statistical analyses.** Inter- and intraassay coefficients of variation were calculated from manufacturer-supplied cytokine standards run with each plate and were typically <0.05 and always <0.10. Cytokine production in the FSL and FRL rats was analyzed using unpaired t tests. Differences were considered statistically significant at $p < .05$.

**Experiment 2: KLH-Specific IFN-γ Production**

**Animals.** Male FSL ($N = 6$) and FRL ($N = 6$) rats bred in the animal research facility at the Wadsworth Laboratories in Albany, New York, were used in this study. These rats were bred from animals obtained from Dr. David Overstreet at the University of North Carolina, Chapel Hill, and the persistence of strain differences in cholinergic sensitivity was ensured before these experiments began by administering oxotremorine and measuring changes in body temperature (16). Animals were housed in a clean facility in a room dedicated to this project. The housing environment was maintained at 22°C with lights on at 0700 and off at 1900. Food and water were available ad libitum. All rats were handled for at least 1 week before the beginning of the study.

**Experimental conditions.** Rats were injected IP with either KLH (300 μg/kg; $N = 8$) or an equivalent weight-adjusted volume of saline ($N = 4$) and returned to their cages. This is the same dose of KLH used in Study 1. Four days after immunization, rats were killed rapidly by CO₂ asphyxiation. This time point was chosen on the basis of previous experiments showing decreases in IFN-γ production by splenocytes from stressed animals 4 days after KLH immunization and stress (29).

**Cell preparation for cytokine analyses.** Splenectomies were removed to culture plates containing sterile complete RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Sigma), 50 μM 2-mercaptoethanol (Sigma), 2 mM glutamine (Sigma), 25 μg/ml gentamicin (BioWhittaker, Walkersville, MD), 1 mM sodium pyruvate (Bio-Whittaker), 1 mM nonessential amino acids (BioWhittaker), and 1% sodium bicarbonate (BioWhittaker), and disaggregated using sterile frosted cover slips. Splenocytes were aspirated, underlayered by Ficoll-Hypaque (Sigma), and spun for 30 min at 1200 rpm. The MNC fraction was removed, washed twice with complete medium, counted in a cell counter (Beckman-Coulter, Fullerton, CA), and resuspended at a density of 2 × 10⁶/ml in complete medium.

**Determination of cytokine production.** MNCs (10⁶/ml) were cultured in a 96-well plate (round-bottom wells, 6 wells per sample) in 200 μl of medium with KLH (150 μg/ml). Plates were incubated in humid 37°C air supplemented with 5% CO₂. After 5 days, supernatants were aspirated and frozen for subsequent analysis. KLH-specific production of IFN-γ was determined using a commercial cytokine ELISA kit (rat IFN-γ Duo-Set kit, R&D, Minneapolis, MN) following the manufacturer’s protocol (with the exception that capture and detecting antibodies, standards, and samples were plated in duplicate 50-μl rather than 100-μl volumes). All samples were analyzed in the same plate to reduce variability. The plate was read at 450 nm (570 nm reference filter) in a spectrophotometer (Molecular Devices). Data are expressed as picograms per milliliter.

**Statistical analyses.** Intraassay coefficients of variation were <0.05 for all standards and samples. Cytokine production in the FSL and FRL rats was analyzed using unpaired t tests. Data from one of the immunized FRL animals suggested that it had failed to respond to KLH (IFN-γ levels for this animal were at the limits of detection).
RESULTS: STUDY 2

Experiment 1: Nonspecific IFN-γ Production

Production of IFN-γ by splenocytes from FSL rats (mean = 407 pg/ml) and FRL rats (mean = 312 pg/ml) did not differ significantly \((t(14) = 1.04, NS)\). As before, the FSL rats (439 g) weighed significantly less than the FRL controls (544 g) at the beginning of the study \((t(14) = 4.05, p < .001)\), and regression analyses revealed no relationship between weight and cytokine production within strain.

Experiment 2: KLH-Specific IFN-γ Production

In rats immunized with KLH 4 days earlier, KLH-stimulated IFN-γ production by FSL splenocytes was significantly lower than FRL production \((t(5) = 3.71, p = .01; \text{Figure 3})\). This effect was sufficiently robust \((r_{\text{effect}} = .86)\) that the power of this analysis \((0.710)\) approached the desirable 0.80 threshold, even with a small number of animals. Cells from saline-injected FSL and FRL rats produced negligible IFN-γ. Moreover, when cells from KLH-immunized animals were cultured for 5 days in the absence of KLH, IFN-γ production was also negligible, a result that agrees with other data from our laboratory showing no detectable increases in serum IFN-γ 4 days after KLH immunization (unpublished data).

As above, the FSL rats (222 g) weighed significantly less than the FRL controls (289 g) at the beginning of the study \((t(5) = 6.21, p < .001)\), and regression analyses revealed no relationship between weight and cytokine production within strain.

DISCUSSION

These studies tested the hypothesis that antigen-specific immunity would be compromised in an animal model of depression. As hypothesized, primary IgM responses to KLH immunization in the FSL rats were significantly lower at each measurement over the course of 2 weeks than in FRL control animals, and although IgG levels did not distinguish the two strains, levels of the IgG2a subtype were significantly lower in the FSL rats, especially on days 11 and 14, when the IgG responses were approaching their peak. This is the first demonstration of differences in antigen-specific antibody production in the Flinders lines and one of the few reports of such immunological differences in any animal model of depression.

The induction of an antibody response to KLH requires T cell involvement, and B cell production of IgG2a specifically is associated with Th1 cell function \((33)\). The marked deficits in IgM and IgG2a production in the absence of reduced IgG1 levels in the FSL rats collectively suggest that Th1 cell regulation of B cell function may be altered. Assessments of Th1 cytokine production in unimmunized FSL and FRL rats revealed no strain differences in IFN-γ levels. This result is in keeping with our previous work showing no strain differences in IL-2 production in unimmunized animals using identical methods \((23)\). In contrast, IFN-γ production by splenocytes from FSL animals immunized with the same dose of KLH used to determine antibody responses were significantly lower than in the FRL controls. Although these results should be interpreted with some caution, given the small number of animals, the robust effect size and the lack of overlap in the IFN-γ data between the two rat strains suggest that this difference is genuine.

Interestingly, there are striking similarities between the current results and the selective reductions of KLH-specific IgM and IgG2a seen in animals undergoing inescapable tail shock stress \((26)\), effects that are thought to be mediated by stress-induced reductions in Th1 cell numbers and IFN-γ production \((26, 35)\). Although we did not examine Th cell numbers in the current study, we have previously reported significantly lower numbers of NK cells in the spleens of FSL rats than in FRL controls under resting conditions \((22)\), and the strain differences in IFN-γ production reported here might be related to strain differences in specific populations of splenocytes.
Body weights among the FSL animals were significantly lower than those of the FRL controls despite the fact that the rats were identical ages. We and others have previously reported this difference (21, 23). Although it is conceivable that strain differences in KLH-specific antibody responses and cytokine production might be related to these differences in body mass, these differences do not seem to influence immune responses in unimmunized animals (23). Moreover, regression analyses involving body mass and either antibody responses or cytokine production within strain show no consistent relationship. Thus, although the body mass differences between the FSL and FRL strains are marked, their immunological differences seem to be influenced by other variables.

In summary, these data extend previous reports of altered immune function in the FSL rats to include in vivo antigen-specific antibody responses and Th1 cytokine production, and these results collectively suggest that Th1 function in the FSL animals may be impaired. The extent to which the types of immunological deficits that exist in the FSL rats translate into increased susceptibility to disease remains to be fully determined, although recent data from our laboratory suggests that the FSL rats are more vulnerable to infection by an intracellular bacterium, defense against which depends on optimal Th1 cell function (36). The current data offer further evidence of the value of the FSL model for illuminating psychological and physiological processes that may contribute to depression-related immunological abnormalities.

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