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Gender- and androgen-related influence on the expression of proto-oncogene and apoptotic factor mRNAS in lacrimal glands of autoimmune and non-autoimmune mice

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Abstract

Our previous studies have shown that the mRNA levels of c-myb, c-myc, bcl-2 and p53 are higher, and partial Fas antigen (i.e. exons 1–2) lower, in lacrimal tissues of female, as compared to male, MRL/lpr mice, which are a model of Sjögren's syndrome. We have also found that this gender-related difference in bcl-2 and c-myb expression appears to be due to the influence of androgens. To extend these findings, we sought to determine: first, whether these gender- and/or hormone-associated variations in mRNA content are unique to MRL/lpr mice, or are also present in lacrimal glands of other murine strains, including autoimmune NZB/NZW F1 (F1) and non-obese diabetic (NOD), as well as non-autoimmune C3H/HeJ (C3H) and BALB/c, mice; and second, whether the levels of these apoptotic factor mRNAs are altered in lacrimal tissues of mice (i.e. testicular feminized (*Tfm*) with dysfunctional androgen receptors, as compared to glandular amounts in their 'normal' controls (i.e. Tabby). Lacrimal tissues were obtained from adult mice, which were either untreated or treated with placebo or testosterone for 21 days. Glands were processed for the analysis of proto-oncogene mRNAs by RT-PCR (at exponential phase of amplification) and data were standardized to the corresponding levels of β-actin mRNA. Our results demonstrate that Fas antigen, Fas ligand, c-myb, c-myc, bcl-2, Bax and p53 mRNAs are present in lacrimal tissues of F1, NOD, C3H, BALB/c, Tabby and *Tfm* mice. The relative levels of Fas antigen mRNA are consistently higher in glands of males, whereas amounts of bcl-2 mRNA are greater in tissues of F1, C3H and BALB/c females. Testosterone administration induced a significant increase in the lacrimal gland content of Bax mRNA, but a striking decrease in the lacrimal tissue level of bcl-2 mRNA in F1 and C3H mice. Lacrimal glands of *Tfm* mice contained elevated amounts of bcl-2 mRNA, as compared to values in tissues of their Tabby controls. In summary, our findings show that fundamental gender-related differences exist in the expression of genes associated with programmed cell death in lacrimal glands of autoimmune and normal mice. In addition, some of these differences may be due, at least in part, to the effect of androgens.

1. Introduction

Sjögren's syndrome is an insidious and currently incurable autoimmune disorder, that occurs almost exclusively in females, and is associated with an extensive lymphocyte accumulation in the lacrimal gland, a precipitous decrease in tear secretion, and severe keratoconjunctivitis sicca (i.e. dry eye) [1]. Recently, our research has shown that androgen (e.g. testosterone), but not estrogen, therapy dramatically suppresses the inflammation in, and stimulates the functional activity of, lacrimal tissue in the MRL/Mp-lpr/lpr (MRL/lpr) female mouse model of Sjögren's syndrome [2–6]. To account for this hormone effect, we have hypothesized that: (a) fundamental, gender-related differences exist that promote inflammation in lacrimal glands of females; and (b) the anti-inflammatory action of

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androgens is a unique, tissue-specific action, that is mediated through a hormone interaction with receptors in epithelial cell nuclei, which then cause an altered expression and/or activity of cytokines, proto-oncogenes and apoptotic factors in the lacrimal gland [7].

In support of this hypothesis, we have found that significant, gender-related differences exist in the magnitude of lymphocyte infiltration [8], and in the levels of cytokine, proto-oncogene and apoptotic factor mRNAs, in lacrimal tissues of MRL/lpr mice [9,10]. Thus, the extent of inflammation in lacrimal glands of females is considerably greater than that in tissues of males [8,11]. In addition, the amounts of IL-1, TNF-α, c-myb, c-myc, bcl-2 and p53 mRNA are higher, and the content of partial Fas antigen (exons 1–2) mRNA is lower, in the inflamed lacrimal glands of female, as compared to male, MRL/lpr mice [9,10]. Furthermore, we have discovered that epithelial cells are the target cells for androgen action in lacrimal tissue [12], and that androgen treatment induces a marked increase in the amount of TGF-β1 protein and Bax mRNA, and a significant decrease in the content of IL-1β, TNF-α, c-myb and bcl-2 mRNAs in lacrimal glands of female MRL/lpr mice [9,10].

However, although these findings are consistent with our hypothesis, it is quite possible that the observed gender-related differences are unique to the MRL/lpr strain. MRL/lpr mice harbor a single mutation in the Fas antigen gene that regulates apoptosis [13–15], and their profile of cytokine, proto-oncogene and apoptotic factor mRNAs may well be distinct from patterns expressed in lacrimal tissues of other models of Sjögren's syndrome or even of 'normal' mice. As an additional consideration, it is also possible that androgen action on cytokine or apoptotic gene expression may not be mediated through specific nuclear receptors in the lacrimal gland, but rather through indirect or 'non-classical' mechanisms (e.g. [16]).

Therefore, to address these possibilities, we sought in the present study to determine whether the gender- and/or hormone-associated variations in apoptotic factor mRNA content are restricted to MRL/lpr mice, or are also present in lacrimal glands of other murine strains, including: (a) NZB/NZW F1 (F1) mice, which are another model of Sjögren's syndrome [17,18], and contain dense lymphoid aggregates in their lacrimal tissue [17,19,20] that are suppressed by testosterone exposure [3]. The autoimmune pathogenesis in these animals involves an inherent B cell defect and differs from that of MRL/lpr mice, which have a basic, immunoregulatory disorder of T cells [21,22]; (b) non-obese diabetic (NOD) mice, which are an established model for type-1 insulin-dependent diabetes mellitus [23], but, because of an extensive lymphocyte infiltration into their lacrimal and salivary glands, have also been proposed as a model for Sjögren's syndrome [24,25]. Androgens are known to prevent pancreatic islet destruction and diabetes in female NOD mice [26]; and (c) non-autoimmune C3H/HeJ (C3H), BALB/c and BALB/b mice. For comparison, we also evaluated the mRNA levels of proto-oncogenes and apoptotic factors in lacrimal tissues of testicular feminized (C57BL/6J-A^{WJ}-Ta^{6J}+/+A^{Tfm}; *Tfm*) mice and their 'normal' controls (i.e. Tabby 6J+; Tabby). *Tfm* mice possess dysfunctional androgen receptors, are completely resistant to androgen influence and are considered to be the most appropriate animal model to assess various androgen-dependent phenomena [27].

Our focus on proto-oncogenes and apoptotic factors in these experiments was prompted by the recognition that these factors: (a) play a critical role in the pathogenesis of autoimmune disease [28]; (b) are inappropriately expressed in Sjögren's syndrome (e.g. Fas antigen, Fas ligand, c-myb, c-myc and bcl-2) [13–15,29–35]; (c) may promote the inflammatory process and the programmed cell death of epithelial cells in exocrine tissues in Sjögren's syndrome [36–41]; and (d) may be influenced by gender and/or regulated by sex steroids (e.g. Fas antigen, c-myc, bcl-2, Bax) in non-ocular sites [42–56].

2. Experimental

2.1. Animals and hormone treatment

Adult, male and/or female F1, NOD, C3H, *Tfm* and Tabby mice were purchased from Jackson Laboratory (Bar Harbor, ME) and BALB/c, BALB/b and C3H/HeN mice were obtained from Taconic Laboratories (Germantown, NY). Animals were maintained in constant temperature rooms with fixed light/dark intervals of 12 h duration. After the onset of disease or at designated ages, age-matched mice were either sacrificed by CO_2 inhalation or were treated with subcutaneous implants of placebo (cholesterol, methyl cellulose, lactose)- or testosterone (10 mg)-containing pellets in the subscapular area. These pellets were purchased from Innovative Research of America (Sarasota, FL) and were designed for the slow, but continual, release of vehicle or hormone over a 3-week period. Following animal sacrifice, exorbital lacrimal glands were removed and processed for molecular biological procedures or acinar epithelial cell isolation. All studies adhered to the Guiding principles in the care and use of animals (DHEW Publication, NIH 80-23).

2.2. Isolation of lacrimal gland acinar epithelial cells

The isolation of acinar epithelial cells from lacrimal glands of C3H/HeN mice was performed by using pre-

viously described techniques [57]. In brief, tissues were rinsed in DMEM (with L-glutamine, 1000 mg D-glucose/l, 100 mg sodium pyruvate/l; Gibco/BRL, Grand Island, NY) containing soybean trypsin inhibitor (0.1 mg STI/ml; Worthington Biomedical, Freehold, NJ), cleared of adherent fascia, minced on ultraviolet light-irradiated dental wax sheets (Polysciences, Warrington, PA) and washed with HBSS (without Ca^{2+} or Mg^{2+}; Gibco/BRL). All DMEM- and HBSS-based media were supplemented with gentamicin (25 μg/ml; Sigma Chemical Co., St. Louis, MO). Glandular fragments were then disrupted through a series of shaking, 37°C incubations in EDTA (0.76 mg/ml; Gibco/BRL), or collagenase (200 U/ml; Calbiochem-Behring, La Jolla, CA), hyaluronidase (698 U/ml; Calbiochem-Behring) and DNase I (10 U/ml; Boehringer Mannheim, Indianapolis, IN) in DMEM- or HBSS-based buffers. These incubations, which were interspersed with several wash procedures, were conducted for approximately 20 min periods in an atmosphere of 95% air–5% CO_2. The resulting tissue digest was filtered sequentially through 500 and 25 μm Nitex mesh (Tetko, Briercliff, NY) and centrifuged at $50 \times g$ for 10 min. The lacrimal cell pellet was resuspended in DMEM containing 20% heat-inactivated FBS (Hyclone, Logan, UT) and centrifuged through a Ficoll 400 (Pharmacia, Piscataway, NJ) step gradient (2–4%) at $50 \times g$ for 15 min. The final cell pellet was resuspended in DMEM and prepared for total RNA extraction.

2.3. Total RNA isolation and reverse transcription

To measure the relative amounts of proto-oncogene and apoptotic factor mRNAs in lacrimal glands and cells of these mice, specific reverse transcription polymerase chain reactions (RT-PCR) were used. Total RNA was isolated from lacrimal (≥2 glands/sample), hepatic, splenic and prostatic tissues, and acinar epithelial cells, by using a TRI reagent (Molecular Research Center, Cincinnati, OH) method, which is based upon a modified acid guanidinium–thiocyanate–phenol–chloroform extraction procedure [58]. The resulting RNA preparations were analyzed by spectrophotometry at 260 nm to measure their concentration and evaluated on 6.6% formaldehyde/1.2% agarose gels to confirm RNA integrity. cDNAs were transcribed from total RNA samples (5 μg) by utilizing 10 units/μl of AMV or MMLV reverse transcriptase, oligo dT priming and either the First-Strand cDNA Synthesis kit from Invitrogen (San Diego, CA) or the Advantage RT-for-PCR Kit from Clontech, according to modifications of the manufacturer's protocol.

PCR amplification (final reaction volume is 25 or 50 μl) of the cDNAs (2–5 μl/sample) was conducted with a Perkin Elmer Cetus GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT) by using 1.0 U Taq DNA polymerase (Gibco/BRL), 0.2 to 1.0 mM each of dATP, dCTP, dGTP and dTTP, PCR buffer (Invitrogen; 10 mM Tris HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) and 0.4 μM of each 5′ and 3′ primer corresponding to mouse Fas antigen, Fas ligand, bcl-2, Bax, c-myb, c-myc, p53, androgen receptor and β-actin mRNA. Primers and oligonucleotide probes were synthesized by National Biosciences (Plymouth, MN) or obtained from Clontech (Palo Alto, CA). With one exception, all primers, probes and PCR conditions were as previously described [9]. The one exception was the preparation of an additional set of Fas antigen primers, which were designed to detect a larger sequence spanning exons 1–3 of Fas antigen mRNA (sense primer: CAGACATGCTGTGGATCTGG; antisense primer: CACAGTGTTCACAGCCAGGA; denaturation is 95°C for 90 s; annealing is 60°C for 90 s; extension is 72°C for 180 s; PCR cycle number is 43; amplified fragment size is 423 bp). The number of PCR cycles chosen for each primer set was determined experimentally, and selected so as to occur during the exponential phase (i.e. before the plateau region) of the amplification.

2.4. Southern blot procedures

After amplification, the PCR products were analyzed on 1.5% agarose gels containing ethidium bromide and a 100 bp DNA molecular weight ladder (Gibco/BRL), in order to verify the anticipated fragment sizes [9]. The amplified cDNA products were then transferred to GeneScreen nylon membranes (Dupont/NEN, Boston, MA) by positive pressure, fixed by UV cross-linking and incubated with specific ^{32}P-labeled probes in a buffer containing 5× SSC, 5× Denhardt's solution, 0.5% SDS and 100 μg sonicated salmon sperm DNA/ml in 0.1% DEPC-treated water. These probes included: (a) internal oligomers (20–22 mer; [9]) that were radiolabeled with γ-^{32}P-dATP (6,000 Ci/mmol) by an end-labeling method with T4 Polynucleotide Kinase (New England Biolabs, Beverly, MA); (b) a 1011 bp fragment of the rat androgen receptor cDNA (corresponding to nucleotide sequence 2827–3837 in the steroid binding domain; gift from Dr. Elizabeth M. Wilson, Chapel Hill, NC), which was purified with a Geneclean kit (BIO 101, La Jolla, CA) and phosphorylated with α-^{32}P-dCTP (3,000 Ci/mmol; NEN/Dupont) by using the random primer extension labeling system (NEN/Dupont) or the random primers DNA labeling system (Gibco/BRL) and (c) a 750 bp Pst-1 fragment of mouse β-actin cDNA (gift from Dr. Lan Hu, Boston, MA) and full length cDNAs (gifts from Dr. Shigekazu Nagata, Osaka, Japan) corresponding to Fas antigen (~1.5 kb) and Fas ligand (~1.7 kb), which were also purified with Geneclean

and labelled with α-^{32}P-dCTP by random priming. Radiolabeled probes were separated from free ^{32}P-nucleotides by filtration through a Sephadex G-25 or G-50 column (Pharmacia, Piscataway, NJ). Following an overnight hybridization at 58°C in an oscillating water bath, the Southern blots were washed and processed for autoradiography by using Kodak XAR X-OMAT film with an intensifying screen at −70°C. Positive and negative control cDNAs were run in parallel, but independent, tubes in all PCR procedures. The positive controls included cDNAs prepared from the liver (Fas antigen, bcl-2), spleen (Fas ligand, Bax, c-myb, c-myc, p53) and prostate (androgen receptor) of adult BALB/c mice, or commercial cDNAs obtained from Clontech (c-myb, c-myc, p53). Negative control tubes contained either no primer sets or no cDNA template.

2.5. Densitometry and statistical analysis

To measure mRNA levels, bands densities in agarose gels and autoradiograms were quantified by the use of an image analysis system and a mathematical treatment of the acquired data. In brief, images of gel internegatives and autoradiograms were captured with an 8 bit (i.e. 256 shades of gray), 144 dpi CCD-72S video camera (Hamamatsu Photonics, Japan). The focal length and aperture settings were optimized for each image and, when necessary, background anomalies were removed in Adobe Photoshop 4.0 by repeating local pixels over the irregular area. The maximum ($\bar{I}_{max}$) and mean ($\bar{I}$) intensities of band absorbance, the sum (Sum) and area (Area) of band pixels, as well as the mean background ($\bar{B}$), were obtained by using the Kodak 1D gel program (Eastman Kodak Company, New Haven, CT). These values were then incorporated into the equation below, in order to calculate a contrast sensitive index of absorbance (i.e. measurement of band density):[1]

$$\left(\frac{256-\bar{B}}{256-I_{max}}\right)\left(\frac{256-\bar{B}}{256-\bar{I}}-1\right)\frac{\text{Sum}}{\text{Area}}$$

For comparison, the net intensity ($\bar{I}-\bar{B}$) of bands was also determined with the Kodak program, as well as an NIH imaging program. All absorbance results were standardized to the corresponding level of β-actin mRNA, as previously described [59,60], and data are reported in terms of the sample mRNA/β-actin mRNA ratio. Statistical evaluation of the data was performed by utilizing Student's unpaired, two-tailed *t*-test.

[1] Sullivan BD, Wickham LA, Sullivan DA. Functional considerations in the measurement of band densities in agarose gels and autoradiograms. In preparation.

3. Results

3.1. Effect of gender on the levels of apoptotic factor mRNAs in lacrimal glands of autoimmune and non-autoimmune mice

The purpose of the following studies was to determine whether gender influences the expression of apoptotic factor mRNAs in lacrimal glands autoimmune and non-autoimmune mice. Lacrimal glands ($n = 2$–6 glands/sample) were obtained from autoimmune F1 (7.5 m old; 6 samples/gender) and NOD (6 m old; $n =$ 5 samples/gender) mice after the onset of disease, and 'normal' C3H (7.8 m old; $n = 6$ samples/gender) and BALB/c (10 weeks old; $n = 5$ samples/gender) mice. Tissues were processed for mRNA analysis by RT-PCR, Southern blot hybridization and densitometry. All data were standardized to the corresponding amounts of β-actin mRNA.

Our results demonstrated that Fas antigen, Fas ligand, bcl-2, Bax, c-myb, c-myc and p53 mRNAs are all present in lacrimal glands of male and female F1, NOD, C3H and BALB/c mice. In addition, as found with MRL/lpr mice [9], the expression of these apoptotic factor mRNAs was significantly influenced by gender. Thus, despite some apparent physiological variability (e.g. Fig. 1, F1, female 1) the content of Fas antigen mRNA was significantly higher in lacrimal tissues of male, as compared to female, F1, NOD, C3H, BALB/c and BALB/b (10–12 weeks old) mice (Fig. 1). Conversely, the mRNA levels of bcl-2 (F1, C3H, BALB/c), c-myb (F1, C3H), c-myc (F1, NOD) and p53 (F1, C3H) were significantly greater in lacrimal glands of female mice (Fig. 2; Table 1).

In striking contrast to these findings was the gender-associated profile of Fas-ligand, c-myb, bcl-2 and p53 mRNAs in the lacrimal glands of NOD mice. In this strain, the relative amount of these mRNAs was significantly greater in tissues of male, as compared to female, mice (Fig. 2, Table 1; Fas ligand mRNA/β-actin mRNA ratio: male = 1.20 ± 0.12, female = 0.53 ± 0.04, $p < 0.005$).

The gender-related differences in proto-oncogene and apoptotic factor mRNA levels could not be attributed to variations in either β-actin mRNA content or in lacrimal gland weight. The amount of β-actin mRNA/5 μg total RNA was typically the same in lacrimal tissues of males and females. Moreover, although the weight of male lacrimal glands was generally 2-fold higher ($p = 0.0001$) than that of females (e.g. F1: M = 37.8 ± 1.1 mg/2 glands, F = 19.0 ± 1.4 mg/2 glands; NOD: M = 46.9 ± 2.3 mg/2 glands,

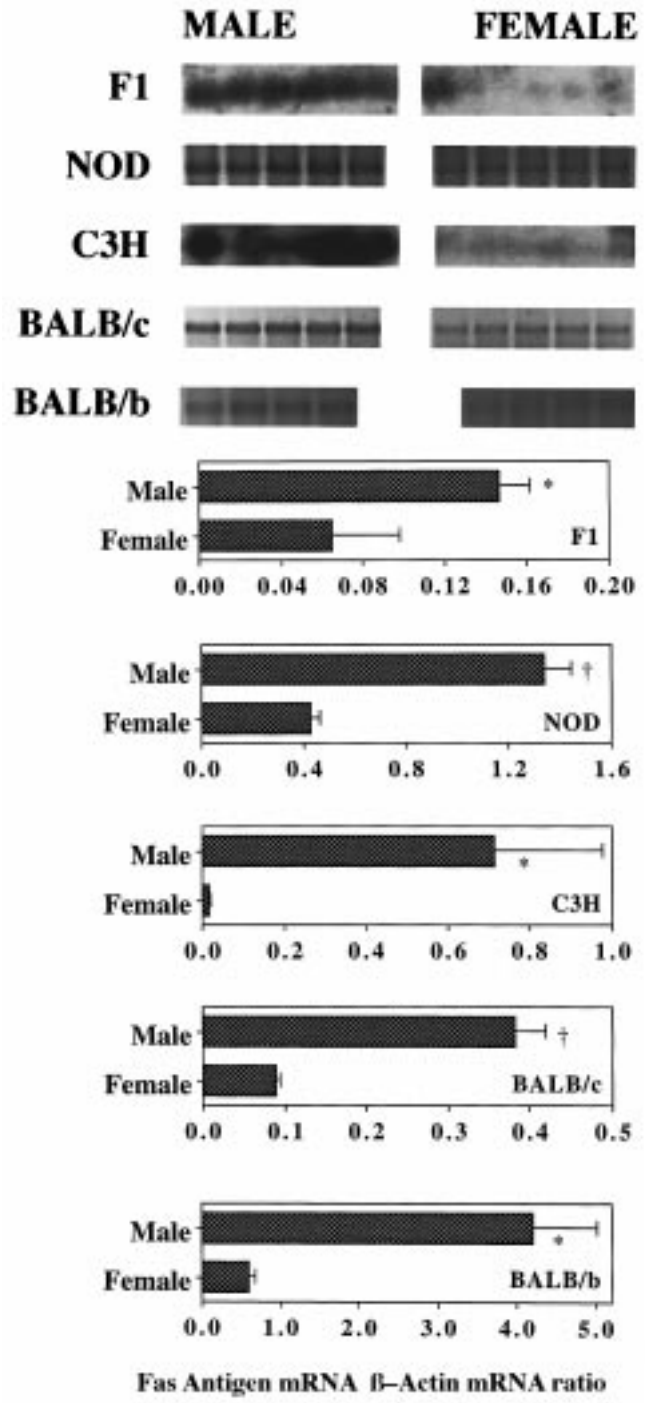

Fig. 1. Effect of gender on the expression of Fas antigen mRNA in lacrimal glands of autoimmune and non-autoimmune mice. Lacrimal tissues were obtained from autoimmune F1 ($n = 6$ samples/gender) and NOD ($n = 5$ samples/gender) mice after the onset of disease, as well as non-autoimmune C3H ($n = 6$ samples/gender), BALB/c ($n = 5$ samples/gender) and BALB/b ($n = 4$ samples/gender) mice. Glands were then processed for the analysis of Fas antigen mRNA (exons 1–2, exons 1–3 or exons 3–7+) by RT-PCR (exponential phase of the amplification), agarose gel electrophoresis and densitometry, and band identity was confirmed by Southern blot hybridization. (Top) Presence of Fas antigen mRNA in lacrimal tissues of male and female mice. The dense band present in the sample from the first F1 female mouse was obtained irrespective of the primer set used for analysis (e.g. exons 1–2, exons 1–3); (bottom) relative levels of Fas antigen mRNA in lacrimal glands of male and female mice. Values reported for the F1 and C3H samples were obtained by analyzing Southern blots, given that the correlative bands on agarose gels were too faint for absorbance measurement. All absorbance results were standardized to the corresponding amount of β-actin mRNA. Columns and bars represent the mean ± S.E. of individual mRNA/β-actin mRNA ratios. *Significantly ($p < 0.05$) greater than the value of the female group; †Significantly ($p < 0.0005$) higher than the value of the female group. It is important to note that the samples of each comparative group (e.g. male vs. female F1) shown in this figure, and all other figures, received identical processing during hybridization, exposure and quantitation. Gels or blots were separated into strips (top) solely for presentation purposes in the figures.

Table 1
Impact of gender on the expression of c-myb, c-myc and p53 mRNAs in lacrimal glands of autoimmune and non-autoimmune mice[a]. *Significantly ($p < 0.05$) greater than the value of the other gender; **Significantly ($p < 0.005$) higher than the value of the male group

	c-myb mRNA/β–actin mRNA		c-myc mRNA/β–actin mRNA		p53 mRNA/β–actin mRNA	
Strain	Male	Female	Male	Female	Male	Female
F1	0.06 ± 0.01	$0.40 \pm 0.11^{*}$	0.53 ± 0.08	$2.01 \pm 0.41^{*}$	0.80 ± 0.11	$1.64 \pm 0.09^{**}$
C3H	0.45 ± 0.12	$0.79 \pm 0.08^{*}$	1.69 ± 0.37	1.65 ± 0.14	0.90 ± 0.19	$1.75 \pm 0.04^{**}$
BALB/c	0.19 ± 0.03	0.18 ± 0.02	0.37 ± 0.02	0.33 ± 0.02	0.44 ± 0.08	0.31 ± 0.07
NOD	$0.63 \pm 0.07^{*}$	0.38 ± 0.07	0.64 ± 0.04	$1.09 \pm 0.09^{**}$	$1.79 \pm 0.09^{*}$	1.37 ± 0.10

[a] Lacrimal tissues were obtained from F1, C3H, BALB/c and NOD mice and processed for RT-PCR, agarose gel electrophoresis and densitometry, as explained in the legend to Fig. 1. The identity of all bands was confirmed by Southern blot hybridization. All absorbance data were standardized to the corresponding level of β-actin mRNA.

F = 26.8 ± 0.8 mg/2 glands; C3H: M = 34.8 ± 1.0 mg/2 glands, F = 17.0 ± 0.8 mg/2 glands), all reverse transcription procedures were conducted with identical amounts (i.e. 5 μg) of total RNA.

Of interest, although apoptotic factor mRNAs may possibly be transcribed in heterogenous cell populations (e.g. lymphocytic, epithelial, endothelial cells), our preliminary research has shown that Fas antigen, Fas ligand, bcl-2, Bax, c-myb, c-myc, p53, as well as androgen receptor, mRNAs are all expressed in acinar epithelial cells of the lacrimal gland in female C3H/HeN mice (Fig. 3).

3.2. Influence of testosterone administration on the expression of proto-oncogene and apoptotic factor mRNAs in lacrimal glands of female F1 and C3H mice

Our past studies have demonstrated that androgens induce a significant increase in the level of Bax mRNA, but a marked decrease in the content of c-myb and bcl-2 mRNAs, in lacrimal glands of female MRL/lpr mice [9]. The objective of the following experiments was to assess whether this hormone action is restricted to the MRL/lpr strain or may also occur in lacrimal tissues of autoimmune F1 and 'normal' C3H mice. Female F1 (8.2 m old) and C3H (8.5 m old) mice ($n = 7$–15 mice/group) were given subcutaneous implants containing either vehicle or testosterone (10 mg) and, following a 21 day period, lacrimal glands were obtained and processed ($n = 2$–6 glands/sample; $n = 4$–6 samples/group) for the analysis of Bax, bcl-2 and c-myb, as well as Fas antigen and Fas ligand, mRNAs.

As shown in Fig. 4, exposure to exogenous testosterone elicited a significant rise in the lacrimal gland content of Bax mRNA, but a striking drop in the lacrimal tissue level of bcl-2 mRNA, in both F1 and C3H mice. In contrast, hormone administration had no consistent influence on the relative amounts of Fas antigen, Fas ligand, c-myb or β-actin mRNAs, compared to contents in glands of placebo-treated controls (data not shown). The effect of androgen treatment on either Bax or bcl-2 mRNA, an action that showed some physiological variability (Fig. 4), could not be explained by fluctuations in lacrimal tissue weight and possible corresponding changes in total RNA levels: testosterone exposure augmented ($p = 0.0001$) the weight of lacrimal glands (F1: placebo = 17.8 ± 0.9 mg/2 glands, testosterone = 40.1 ± 2.3 mg/2 glands; C3H: 16.7 ± 0.5 mg/2 glands, testosterone = 24.4 ± 0.9 mg/2 glands), but all mRNA analyses were made with analogous levels of total RNA.

3.3. Impact of androgen receptor dysfunction on the relative amounts of apoptotic factor mRNAs in the lacrimal gland

Given the impact of gender and androgen treatment on the expression of proto-oncogene and apoptotic factor mRNAs in lacrimal tissue, the following study was designed to determine whether these influences may depend upon the presence of a functional androgen receptor. Accordingly, lacrimal glands ($n = 1$–2 glands/sample) were obtained from *Tfm* (80 ± 2 days old; $n = 10$) and Tabby control (81 ± 3 days old; $n = 4$) mice and processed for the evaluation of Fas antigen, Fas ligand, bcl-2, Bax, c-myb, c-myc, p53, androgen receptor and β-actin mRNAs.

Comparison of mRNA levels in these tissues demonstrated that the absence of functional androgen receptors in *Tfm* mice was associated with a significant increase in the expression of bcl-2 mRNA (Fig. 5). In contrast, no consistent differences were observed between the relative amounts of Fas antigen, Fas ligand, Bax, c-myb, c-myc, p53 or β-actin mRNAs in glands from *Tfm* and control mice (data not shown). In these experiments, as shown in Fig. 5, androgen receptor mRNA was undetectable in lacrimal tissues of *Tfm* mice. A similar loss of androgen receptor mRNA has also been observed in other tissues of *Tfm* mice [61,62].

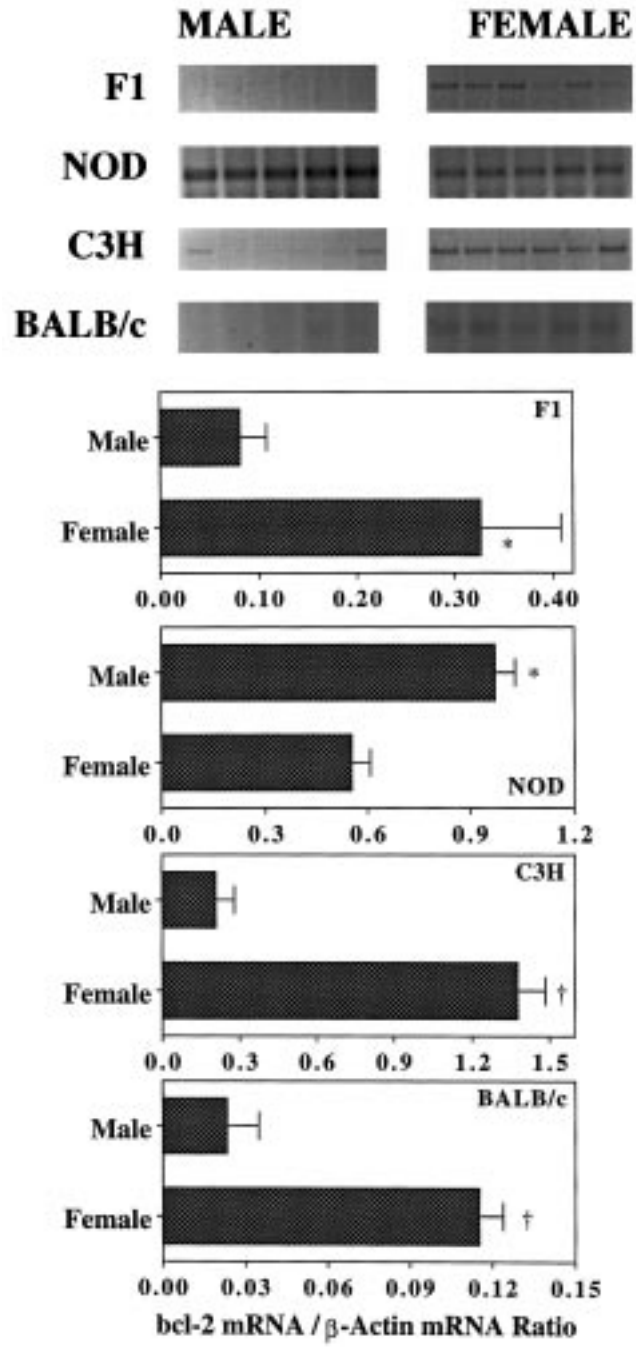

Fig. 2. Influence of gender on the expression of bcl-2 in lacrimal tissues of F1, NOD, C3H and BALB/c mice. After obtaining lacrimal glands from animals, tissues were processed for RT-PCR, agarose gel electrophoresis and densitometry, and data were standardized as explained in the legend to Fig. 1. The identity of all bands was verified by Southern blot hybridization. (Top) Presence of bcl-2 mRNA in lacrimal glands of male and female mice. Note that in some samples (e.g. NOD) an additional lower band, which was not quantitated, is apparent. This band may have been due to alternative splicing of the bcl-2 gene (e.g. [82]); (bottom) relative levels of bcl-2 mRNA in lacrimal tissues of male and female mice. *Significantly ($p < 0.05$) greater than the value of the other gender; †Significantly ($p < 0.0005$) higher than the value of the male group.

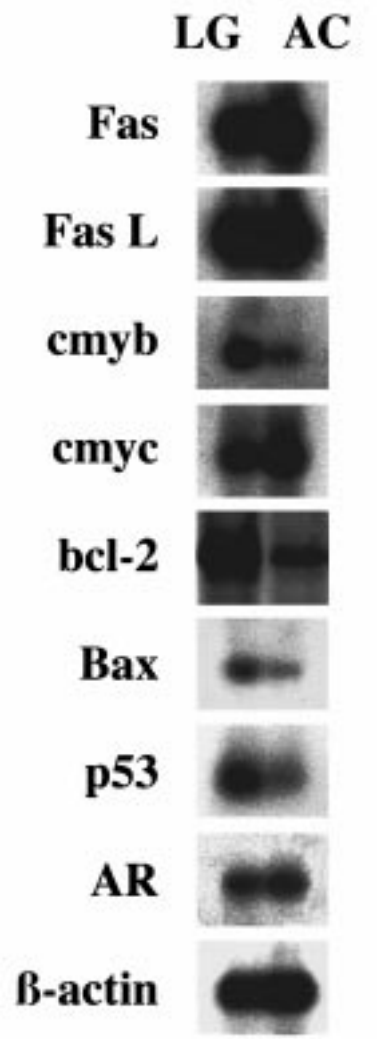

Fig. 3. Presence of Fas antigen (exons 3–7+), Fas ligand, bcl-2, Bax, c-myb, c-myc, p53, androgen receptor (AR) and β-actin mRNAs in whole (LG), and isolated acinar epithelial cells (AC) from, lacrimal glands of female C3H/HeN mice. Lacrimal tissues were obtained from C3H/HeN mice ($n = 20$) and either saved intact ($n = 3$ glands) or utilized for the isolation of acinar cells ($n = 37$ glands; yield is 6×10^7 cells). Glands and cells were then processed for RT-PCR and Southern blot hybridization.

4. Discussion

The present study shows that significant, gender- and strain-related differences exist in the expression of genes associated with programmed cell death in lacrimal glands of autoimmune and 'normal' mice. Thus, the levels of Fas antigen mRNA were significantly greater in lacrimal tissues of male, as compared to female, F1, NOD, C3H, BALB/c and BALB/b mice, whereas the amounts of bcl-2 (F1, C3H, BALB/c), c-myb (F1, C3H), c-myc (F1, NOD) and p53 (F1, C3H) mRNAs were significantly higher in lacrimal glands of female mice. These gender-related variations are analogous to those previously observed in lacrimal tissues of

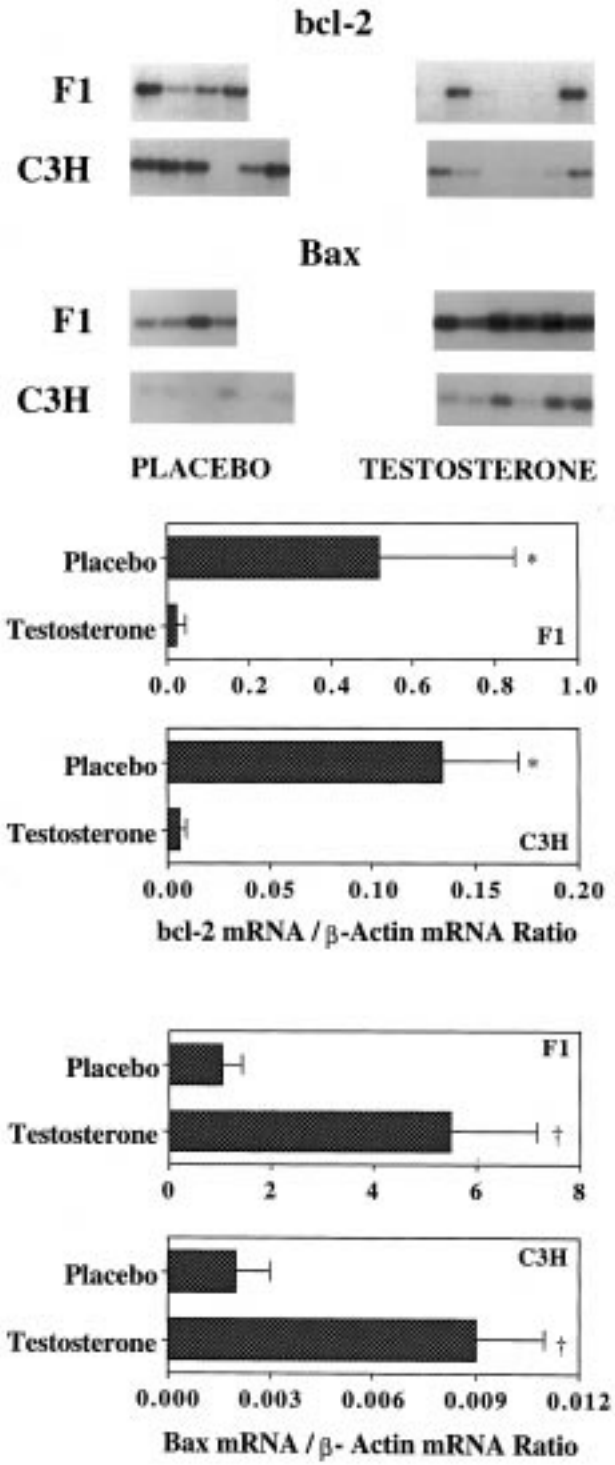

Fig. 4. Effect of placebo or testosterone treatment on the expression of bcl-2 and Bax mRNAs in lacrimal glands of female F1 and C3H mice. Animals were treated with placebo- or testosterone-containing compounds for 3 weeks, and then lacrimal tissues ($n = 4$–6 samples/group) were obtained and processed for mRNA analysis. Absorbance values of bands in Southern blots were measured and standardized as described in the legend to Fig. 1. (Top) Expression of bcl-2 mRNA in lacrimal glands of placebo- and testosterone-treated mice; (bottom) relative amounts of bcl-2 mRNA in lacrimal tissues of mice administered vehicle or hormone. *Significantly ($p < 0.05$; 1 tailed in F1) greater than the value of the testosterone-treated group; †Significantly ($p < 0.05$; one-tailed in F1) higher than the value of the placebo-treated group.

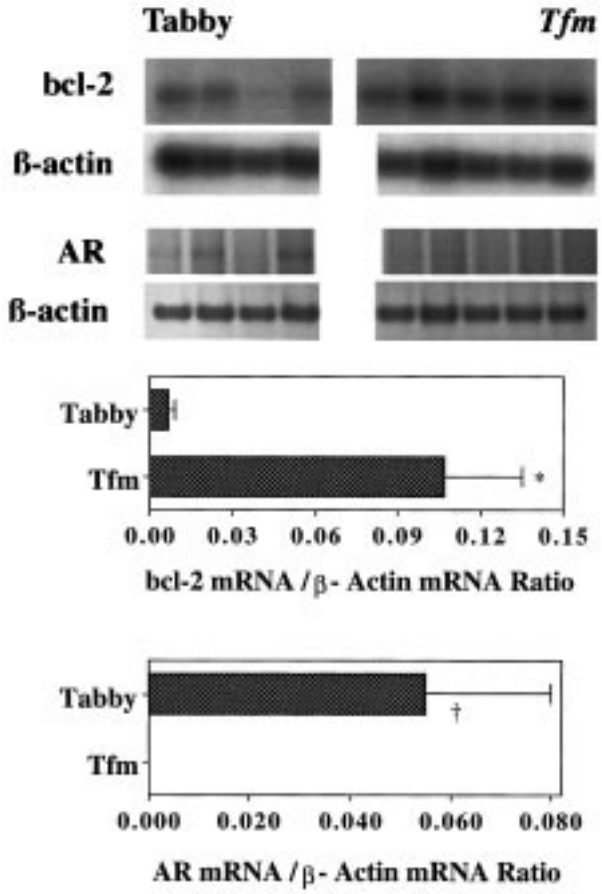

Fig. 5. Relative levels of bcl-2 and androgen receptor mRNAs in lacrimal tissues of male *Tfm* and Tabby control mice. Lacrimal glands ($n = 4$–5 samples/group) were obtained from untreated animals and processed for mRNA analysis. Absorbance values of bands in agarose gels (androgen receptor; AR) and Southern blots (bcl-2) were determined and standardized to corresponding β-actin mRNA amounts, as described in the legend to Fig. 1. (Top) Expression of bcl-2 and corresponding β-actin (Southern blot with bcl-2, gel with AR) mRNAs in lacrimal tissues of Tabby and *Tfm* mice; (bottom) relative levels of bcl-2 and AR mRNA in lacrimal glands of Tabby and *Tfm* mice. *Significantly ($p < 0.05$) greater than the control value; †Significantly ($p < 0.05$) higher than the Tfm value.

MRL/lpr mice [9], but differ markedly from the profile of Fas ligand, bcl-2, c-myb and p53 mRNAs expressed in glands of NOD mice. An additional finding in the current investigation was that androgens appear to regulate the content of bcl-2 and Bax mRNA in lacrimal tissue of both autoimmune and non-autoimmune mice.

Our observation that gender-associated differences occur in the expression of proto-oncogene and apoptotic factor mRNAs in lacrimal glands of a variety of murine strains was particularly intriguing for at least two reasons. First, despite the different origins of their autoimmune disorders, both MRL/lpr [9] and F1 mice had similar, gender-related profiles of Fas antigen, bcl-2, c-myb, c-myc and p53 mRNAs in their lacrimal tissues. Given that both of these strains are models of Sjögren's syndrome [11,17–19,21], and that, as in humans [1], the severity of their disease is far worse in females [21], it is possible that their apoptotic factor mRNA pattern may play a role in promoting lacrimal gland inflammation. In support of this possibility, other investigators have linked alterations in Fas antigen [13–15,37], bcl-2 [33,39], c-myb [29,30] and c-myc [31,32] levels to the etiology and/or development of various non-ocular immune abnormalities in Sjögren's syndrome. A second intriguing aspect of our findings is that the variations between males and females in the expression of Fas antigen and bcl-2 mRNAs may not solely reflect susceptibility to, or the presence of, inflammatory disease in lacrimal tissue. Rather, given that gender-associated differences exist in the levels of these apoptotic factor mRNAs in glands of non-autoimmune mice, it may be that these variations also represent fundamental differences in the physiology of lacrimal tissue. If so, this fact would not be surprising, considering that gender exerts a significant influence on numerous structural and functional indices in the lacrimal glands of multiple species [63].

In contrast to our results with MRL/lpr [9], F1, C3H and BALB/c mice, NOD mice possessed a very different gender-related profile of proto-oncogene and apoptotic factor mRNAs in their lacrimal tissues. Thus, the relative levels of Fas ligand, bcl-2, c-myb and p53 were significantly higher in glands of males, as compared to female, mice. This strain difference, though, may be somewhat unique and may possibly reflect the altered immune and endocrine status of NOD mice. To explain, the NOD strain has been proposed as a model for Sjögren's syndrome [24,25], but this proposition may be incorrect. One of the most striking features of Sjögren's syndrome is the female prevalence in the incidence of disease [1], and this predominance, in turn, translates into a far greater frequency of lacrimal gland inflammation in females, as compared to males [1]. These characteristics are true of both humans with, and animal models (e.g. MRL/lpr mice) of, Sjögren's syndrome [1,8,11]. However, although the incidence of the autoimmune diabetic disease in NOD mice is considerably higher in females [64,65], the extent of lacrimal tissue inflammation is significantly more pronounced in males [8,28]. Consequently, this lymphocyte accumulation could represent the source of increased Fas ligand, bcl-2, c-myb and p53 mRNA levels, given that these factors are expressed by immune cells [13–15,29–31,33–35,66]. In further support of this hypothesis, preliminary studies indicate that lymphoid aggregates in lacrimal glands of male NOD mice contain bcl-2 protein (Ikuko Toda, unpublished), whereas this proto-oncogene is found primarily in epithelial cells of MRL/lpr lacrimal tissue [9]. Of interest, bcl-2 dysregulation (e.g.

in lymphocytes) has been suggested as a potential cause of the NOD mouse autoimmune disease and the genetic region linked to the induction of NOD periinsulitis occurs in the vicinity of the bcl-2 gene in chromosome 1 [67].

Another possible reason why the amounts of several apoptotic factor mRNAs are different in lacrimal glands of NOD mice, relative to other strains, is their altered endocrine status. These NOD animals are a model for insulin-dependent diabetes [23] and diabetes causes a significant impairment in the structure, function and androgen responsiveness of lacrimal tissue [68]; these sequelae appear to be due, at least in part, to insulin deficiency [57]. Diabetes may also modify the expression of bcl-2 [67,69], c-myc [70,71] and p53 [72], as well as influence the apoptotic resistance of T cells [67,69], which appear to be involved in the progression of autoimmune disease in NOD mice [65]. Consequently, it is quite possible that the distinctive, gender-related pattern of proto-oncogene and apoptotic factor mRNAs in lacrimal glands of NOD mice may be a result of their atypical (i.e. for Sjögren's syndrome) inflammation and diabetic condition.

With regard to androgens, our research showed that testosterone increased the content of Bax, but decreased the level of bcl-2, mRNA in lacrimal tissues of female F1 and C3H mice. This hormone effect, which was also found in MRL/lpr mice [9], may well represent an androgen action on proto-oncogene expression within epithelial cells. This interpretation is prompted by several considerations. First, the testosterone regulation occurred in both autoimmune and non-autoimmune strains, suggesting that the hormone influence is not mediated primarily through alterations in the lymphoid compartment (e.g. suppression of inflammation). Second, we identified Bax and bcl-2 mRNAs in epithelial cells from 'normal' lacrimal glands. Third, epithelial cells are the androgen target cells in lacrimal tissue [12]. Fourth, androgens also appear to modulate Bax and bcl-2 levels in epithelial cells of other tissues [47,53,73].

However, the mechanism by which androgens might control Bax and bcl-2 mRNA expression in epithelial cells is unclear. Indeed, this lack of clarity is particularly profound in terms of the androgen–Bax interaction. No gender-associated differences were observed in the amount of Bax mRNA in lacrimal glands of any murine strain. Moreover, no difference in Bax mRNA content was found between lacrimal tissues of *Tfm* mice and their Tabby controls. If androgens directly control Bax expression, evidence of this activity should theoretically have been present in the *Tfm*–Tabby comparison. *Tfm* mice contain a single base deletion in the amino-terminal domain of androgen receptor mRNA, thereby causing a frame shift in translation and resulting in the premature termination of androgen receptor synthesis and the loss of DNA- and steroid-binding domains [27]. In addition, the androgen production in these mice is severely attenuated [74], which deficiency, when combined with the androgen receptor defect, would serve to inhibit both classical and non-classical effects of androgens. One possible explanation for our findings is that the androgen modulation of Bax mRNA levels in the lacrimal gland may be gender-dependent. Thus, certain effects of androgens and other sex steroids are known to occur only in males or females [75], and it may be that the androgen–Bax interrelationship is limited to females. As concerns bcl-2, the androgen down-regulation of this proto-oncogene in lacrimal tissue may account for the low bcl-2 mRNA content in glands of male MRL/lpr [9], F1, C3H and BALB/c mice, as well as the high bcl-2 mRNA expression in tissues of *Tfm* mice.

In contrast to Bax and bcl-2, testosterone had no influence on the mRNA levels of Fas antigen in lacrimal glands of MRL/lpr [9], F1 and C3H mice, despite the striking gender-related differences found in the expression of this apoptotic factor: in all strains examined, the Fas antigen mRNA content was significantly higher in lacrimal tissues of males than those of females. This lack of androgen effect was also indicated by the absence of any difference in Fas antigen mRNA levels between glands of *Tfm* and Tabby mice. It may be that Fas antigen mRNA expression in lacrimal tissue is regulated by female sex steroids (i.e. estrogens and/or progestins), which could account for the gender effect, but this possibility remains to be explored.

In our studies, we also observed that Fas antigen, Fas ligand, bcl-2, Bax, c-myb, c-myc and p53 mRNAs are all expressed in acinar epithelial cells of lacrimal tissues from 'normal' mice. If translated, these factors, which are also present in epithelial cells of other sites [32,36–40,45,47,50–54,76–79], might play a role in the growth and proliferation of acinar cells in the lacrimal gland. In addition, given that high levels of Fas antigen and Bax, and low titers of bcl-2, promote programmed cell death [80,81], our findings would suggest that epithelial cells in lacrimal tissues of males (i.e. high Fas antigen, low bcl-2) and androgen-treated females (i.e. high Bax, low bcl-2) may be susceptible to an enhanced rate of apoptosis and turn-over.

Lastly, our research demonstrated that the analysis of gel and autoradiogram bands was greatly enhanced by use of the index described in the Experimental section. This mathematical formula[1], which maps image data onto a sigmoid-like curve (an exponential between two linear saturation regions), provided a degree of sensitivity not available with a simple linear function. This index was able to better evaluate the relative contrast of a band, and to more clearly ident-

ify the presence (e.g. gender influence on c-myc in F1 mice and on c-myb and p53 in C3H mice) or absence (e.g. c-myb expression in Tfm versus Tabby mice) of differences between apparently similar bands. In addition, the index: (a) diminished the effect of assymetric light infiltration in internegatives of agarose gels, a condition that interferes with band absorbance measurement in both the Kodak and NIH image programs; and (b) minimized the subjectivity of band analysis, a condition that limits the efficacy of the NIH imaging software[1]. When light infiltration was not a factor, the index and the net intensity typically yielded analogous results.

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