

HETEROGENEITY OF HUMAN T-CELL GROWTH FACTOR(S) DUE TO VARIABLE GLYCOSYLATION*

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Abstract—T-cell growth factor (TCGF) prepared from human tonsil cells was shown to separate into multiple peaks of activity on isoelectric focusing gels and SDS-PAGE. Treatment with neuraminidase and glycosidases or inhibition of glycosylation, however, demonstrated that this heterogeneity was primarily due to sialylation and, to a lesser extent, differences in other sugar residues. In contrast, TCGF prepared from a human T-leukemia cell line was uniform in charge and size and indistinguishable from the asialo form of tonsil-derived activity. Both sialylated and asialo-TCGF mediated continuous *in vitro* T-cell proliferation over successive passages. Thus, the evidence is consistent with a variably glycosylated protein as the entity responsible for T-cell growth.

INTRODUCTION

Recent studies have indicated that the initiation and maintenance of T-cell proliferation is mediated by a soluble T-cell growth factor(s) [TCGF(s)] released from lectin- or antigen-activated T-cells (Morgan *et al.*, 1976; Ruscetti *et al.*, 1977; Gillis *et al.*, 1978; Smith *et al.*, 1979). Preliminary biochemical characterization of human TCGF revealed that activity was present in a protease-sensitive 15,000–20,000 mol. wt entity that did not appear to contain carbohydrate. When the material was subjected to ion-exchange chromatography and isoelectric focusing, however, the activity appeared heterogeneous (Mier & Gallo, 1980; Gillis *et al.*, 1980). It thus remained unclear whether TCGF activity resided in several distinct proteins or in a single protein that had undergone post-translational modification or degradation. Furthermore, because the activity could not be ascribed to a single molecule, some investigators attributed T-cell growth to a class of biologically active molecules, rather than to a distinct entity (Watson & Mochizuki, 1980). If this were true, then one would anticipate that T-cell proliferation results from a complex interaction between antigen/lectin and perhaps several soluble factors.

An alternate possibility was that the apparent heterogeneity of TCGF was due to variable

glycosylation. In this report we demonstrate that while TCGF activity separated into three distinct molecular components on isoelectric focusing (IEF), this heterogeneity was due to variable glycosylation and sialylation. Enzymatic removal of carbohydrate residues, production of TCGF in the presence of 2-deoxy-D-glucose, and production of TCGF from a human T-leukemia cell line all yielded activity that, within the limitations of the methodology, was uniform with respect to size and charge. Both asialo and sialylated TCGF mediated continuous T-cell growth *in vitro*, indicating that although sialylation contributes to significant charge differences it does not appear to be required for biological activity. Since continuous T-cell proliferation was promoted by TCGF that appeared to be a single entity on the basis of size and charge, the results indicate that T-cell growth can be attributed to a direct interaction of TCGF with activated T-cells, rather than to a more complex mechanism involving several soluble factors.

MATERIALS AND METHODS

TCGF production

TCGF was prepared from human tonsil cells and a human T-leukemia cell line (JURKAT) (Kaplan *et al.*, 1976). Cells were cultured at 4×10^6 cells/ml in serum-free RPMI 1640 medium (GIBCO Diagnostics, Grand Island, New York) with 1 μ g/ml phytohemagglutinin (Wellcome Reagents, England) and phorbol

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myristic acetate (50 ng/ml, Consolidated Midlands Corp., Brewster, New York). Irradiated (5000 rad) lymphoblastoid cells (2.5×10^5 cells/ml) (DAUDI) (Tsubota *et al.*, 1977) were added to the tonsil cells to boost TCGF release (Ruscetti *et al.*, 1980). After 24–48 hr at 37°C the supernatants were harvested, filtered (0.45 μ), and stored at 4°C. Inclusion of phorbol myristic acetate (which had no effect on the electrophoretic behavior of TCGF released in its presence and a negligible direct or synergistic effect in the TCGF assay) resulted in a four-fold (tonsils) to 20-fold (JURKAT cell line) increase in release of TCGF activity. The typical yield of TCGF in supernatants of stimulated tonsil cells was 10 units/ml and for the JURKAT cell line, 80 units/ml (see definition of units under *TCGF assay*).

TCGF assay

TCGF activity was determined as previously described (Gillis *et al.*, 1978) by the TCGF concentration-dependent stimulation of proliferation of a cloned murine cytotoxic T-lymphocyte line (CTLL-2, subclone 15H) (Baker *et al.*, 1979). CTLL proliferation, as indicated by [^3H]Tdr incorporation, was determined during the last 4 hr of a 24-hr culture period in the presence of serial two-fold dilutions of a standard TCGF preparation and the experimental samples. The TCGF concentration present in each sample was quantified by probit analysis and expressed as units per millilitre. The standard TCGF preparation, which was arbitrarily assigned a value of 1 unit/ml, routinely yielded 50% of the maximal CTLL [^3H]Tdr incorporation at a dilution of 1:10. The concentration of TCGF in an experimental sample was thus determined by the following formula:

$$[\text{TCGF}] = \frac{\text{experimental reciprocal titer}}{\text{standard reciprocal titer}}$$

where a titer is defined as the dilution that yields 50% of the maximum CTLL [^3H]Tdr incorporation. As an example, if an experimental sample yielded 50% of the maximal CTLL [^3H]Tdr incorporation at a dilution of 1:1200, and the standard preparation yielded 50% of the maximal [^3H]Tdr incorporation at a dilution of 1:10, the experimental sample contained 120 units/ml. By comparing the experimental titers to a standard in each assay, the small variations ($\pm 20\%$) from assay to assay were eliminated.

The activity of various TCGF fractions was also determined with an analogous assay using phytohemagglutinin-stimulated human T-cell blasts. The blasts (3 days post-stimulation) were grown in media containing TCGF (Sephadex G-100 column) for a period of 8–10 days. At this point, the proliferative response to phytohemagglutinin was negligible and the cells were completely dependent on TCGF for growth. TCGF activity was measured as discussed earlier except that the cultures were pulsed with [^3H]Tdr for the last 4 hr of a 48-hr period.

In the chromatographic and electrophoretic procedures described later, any effect of potentially toxic buffer constituents (e.g. NaCl, NaN_3 , sodium dodecyl sulfate, ampholines) on the cells in the microassay was eliminated by a large initial dilution of the samples prior to their inclusion in the assay. This initial dilution was made possible by starting with large quantities of TCGF (100–10,000 units) derived from several litres of conditioned media.

Gel filtration

Pooled supernatants were concentrated 1000-fold using an Amicon (Amicon, Lexington, Massachusetts) CH4 Hollow Fiber Concentrator (HIP5 cartridge) and an Amicon Model 3 or 52 stirred cell with YM-5 membranes. Less than 5% of the TCGF activity was lost during this process. Concentrated material (1% of the column volume) was chromatographed on Sephadex G-100 superfine (Pharmacia, Piscataway, New Jersey) in 10 mM Tris, pH 7.5, 0.5 M NaCl, and 0.02% NaN_3 . As described previously, TCGF activity eluted as a symmetrical peak in a position corresponding to that of a globular protein of 19,000–20,000 daltons (Gillis *et al.*, 1980). Recovery was typically 75–80%, with a 12-fold purification.

IEF

Focusing was performed at 4°C on 8 × 180 mm cylindrical gels (7% acrylamide; 6.25% dilution of Pharmlyte, Pharmacia, New Jersey) at 1000 V for 10–14 hr. The gels were cut into 2-mm slices and eluted (72–96 hr) in 1 ml of 10 mM Tris, pH 7.5, 0.15 M NaCl, 1 mg/ml polyethylene glycol 6000. Each fraction was assayed to determine the level of TCGF activity. No detectable phytohemagglutinin, as judged by direct mitogenicity on murine splenocytes, was found in any of the fractions that contained TCGF activity. In addition, lymphocyte activating

factor which co-migrated on gel filtration with TCGF, focused at a considerably lower pH (Smith *et al.*, 1980).

Enzyme treatment

Samples of TCGF were incubated at 37°C for 4 hr with and without 0.5–5.0 μ neuraminidase (*C. perfringens*, Sigma, Saint Louis, Missouri, No. N9130) or for 18 hr with various amounts of a glycosidase mixture from *D. pneumoniae* containing neuraminidase, endo- β -*N*-acetylglucosaminidase D, β -galactosidase, β -*N*-acetylglucosaminidase, and endo- α -*N*-acetylgalactosaminidase (Kobata, 1979). Both enzyme preparations contained extremely low to undetectable amounts of proteolytic activity.

Inhibition of glycosylation

Tonsil cells were stimulated with phytohemagglutinin, phorbol myristic acetate and DAUDI cells in the presence of 2-deoxy-D-glucose (0.5–10 mM). The resulting supernatants were harvested, concentrated and then examined by isoelectric focusing.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

TCGF preparations were electrophoresed on 0.6 \times 12 cm gels (12% acrylamide) according to the method of Laemmli (1970). Lyophilized samples were dissolved in the prescribed buffer (2% SDS, no 2-mercaptoethanol) and heated at 60°C for 15 min. After electrophoresis the gels were cut into 1-mm slices, each slice was eluted in 0.25 ml RPMI 1640, 10% fetal calf serum (with 10 mg/ml bovine albumin to absorb SDS) and assayed for TCGF activity. Typical recovery of activity was of the order of 30% with the bulk of the loss occurring during lyophilization of the gel samples.

TCGF-dependent T-cell growth

CTLL-2 subclone 15H cells (Baker *et al.*, 1979) were cultured at 2×10^4 cells/ml in DMEM (GIBCO) supplemented with 10% FCS, 50 μ g/ml gentamicin, 50 μ /ml penicillin G, 300 μ g/ml L-glutamine, and 2 μ /ml TCGF activity (determined by the TCGF microassay) isolated from IEF and SDS-PAGE. Viable cell concentrations were determined at 3-day intervals by means of a Coulter Counter (Coulter Electronics, Hialeah, Florida) in conjunction with trypan blue dye exclusion, after which the cultures were re-seeded to 2×10^4 cells/ml.

RESULTS

IEF patterns

When the active fractions of tonsillar-derived material (single or multiple donors) isolated by gel chromatography were pooled and subjected to IEF using a broad (pH 3–10) or narrow (pH 6.5–9) pH gradient, three major peaks of activity were observed (Fig. 1A). The approximate pI values obtained from several experiments were 8.2 (peak a), 7.9 (peak b), and 7.6 (peak c). Similar results were obtained with a 10-fold greater sample size using a flat bed apparatus with Sephadex G75 as support. In both cases total recovery ranged from 40 to 70%. Essentially the same profile was obtained with material focused in gels containing 4 or 6 M urea, which suggested that the peaks did not arise as a consequence of noncovalent interactions between TCGF and contaminating molecules. In

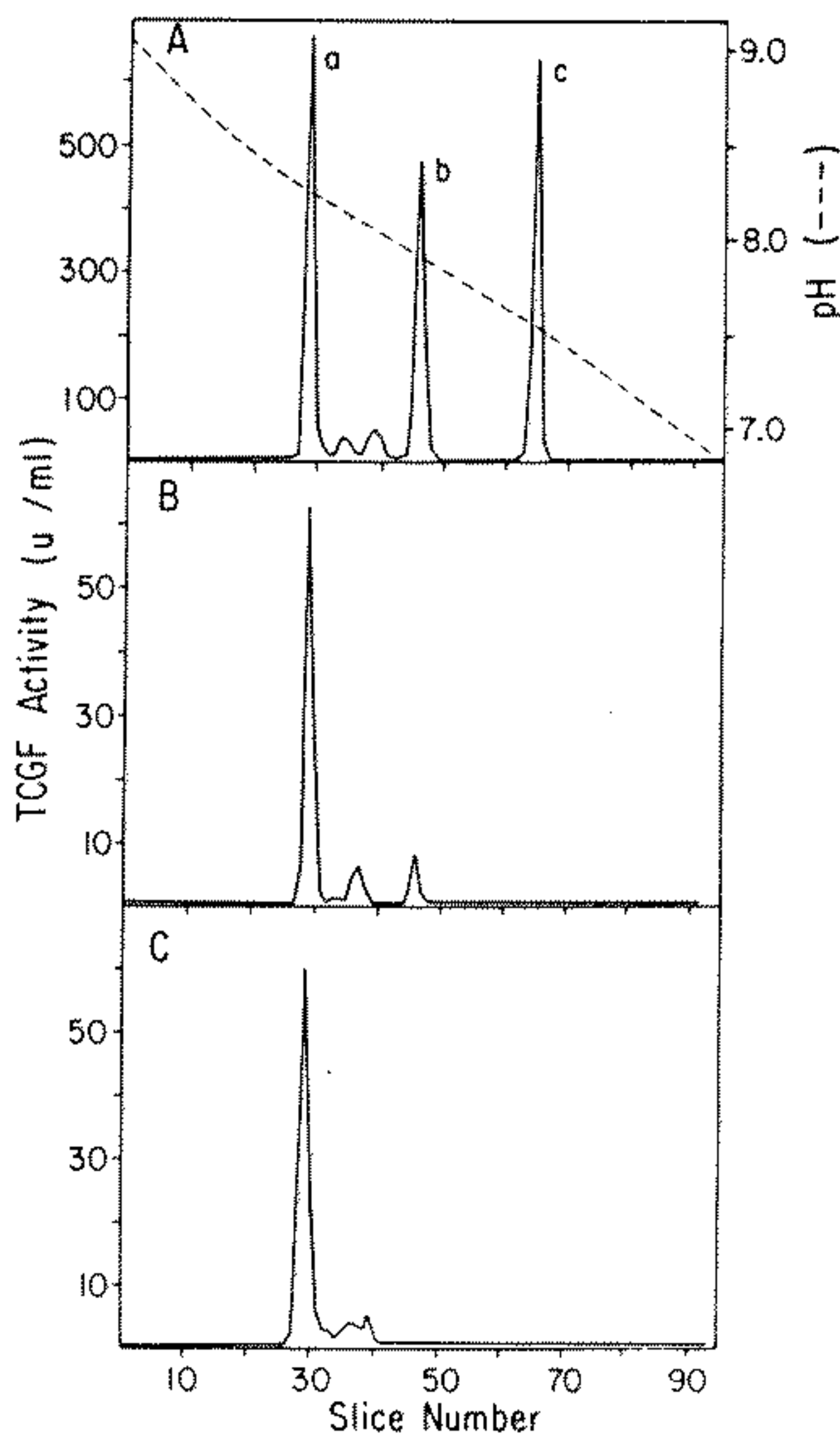


Fig. 1. IEF gels (Pharmolyte, pH 6.5–9). (A) Sephadex G100 pool of tonsil-derived TCGF (4000 units TCGF loaded). (B) Sephadex G100 pool of JURKAT cell line-derived TCGF (120 μ TCGF loaded). (C) Sephadex G100 pool of tonsil-derived TCGF after incubation with neuraminidase (120 μ TCGF loaded). The representative pH gradient illustrated in A was determined using a parallel gel (no sample).

contrast to the IEF profile of activity obtained with TCGF derived from normal human tonsil cells, TCGF derived from the human T-leukemia cell line, JURKAT (Fig. 1B), gave rise to only a single major peak of activity corresponding to peak a (pI 8.2) of the tonsil-derived preparation.

The effect of enzyme treatment

Glycosylation with a variable sialic acid content was one explanation that could account for the heterogeneity of the material derived from normal human cells. To test this possibility, tonsil-derived TCGF isolated by gel chromatography was incubated with and without neuraminidase prior to IEF. As depicted in Fig. 1C, the three peaks of TCGF activity present in the untreated sample were replaced by a single peak after neuraminidase treatment. This peak consistently corresponded to the position of the high pI (8.2), peak a, of the untreated sample, and of the pI of the JURKAT material. The same result was obtained with a mixture of endo- and exoglycosidases and neuraminidase from *D. pneumoniae*. Assays of TCGF following either enzyme treatment revealed no detectable loss of activity.

The shift of TCGF activity to a higher pI value was consistent with the removal of negatively charged *N*-acetyl neuraminic acid from peaks b and c. To ensure that the activities in peaks b and c were actually converted to the higher pI value and not merely lost or destroyed, TCGF eluted from these positions was treated with neuraminidase and re-electrophoresed. As shown in Fig. 2A and B, peak b shifted to the higher pI value after treatment with neuraminidase. Similarly, peak c also shifted to the higher pI value (Fig. 2C and D). Furthermore, when TCGF isolated from peak c was incubated with small amounts of neuraminidase there was a shift of activity to an intermediate position corresponding to peak b, followed by a shift to the peak a position with additional enzymatic treatment. There was no shift in the pI of peak a, or of the major peak of JURKAT material after neuraminidase treatment. As confirmation of the presence of sialic acid, tonsil-derived TCGF isolated by gel chromatography was treated under conditions (0.075 N HCl/75°C/1 hr) designed to hydrolyze terminal *N*-acetyl neuraminic acid (Spiro, 1966). As with neuraminidase, this procedure resulted in conversion of the activity in peaks b and c on IEF gels to peak a.

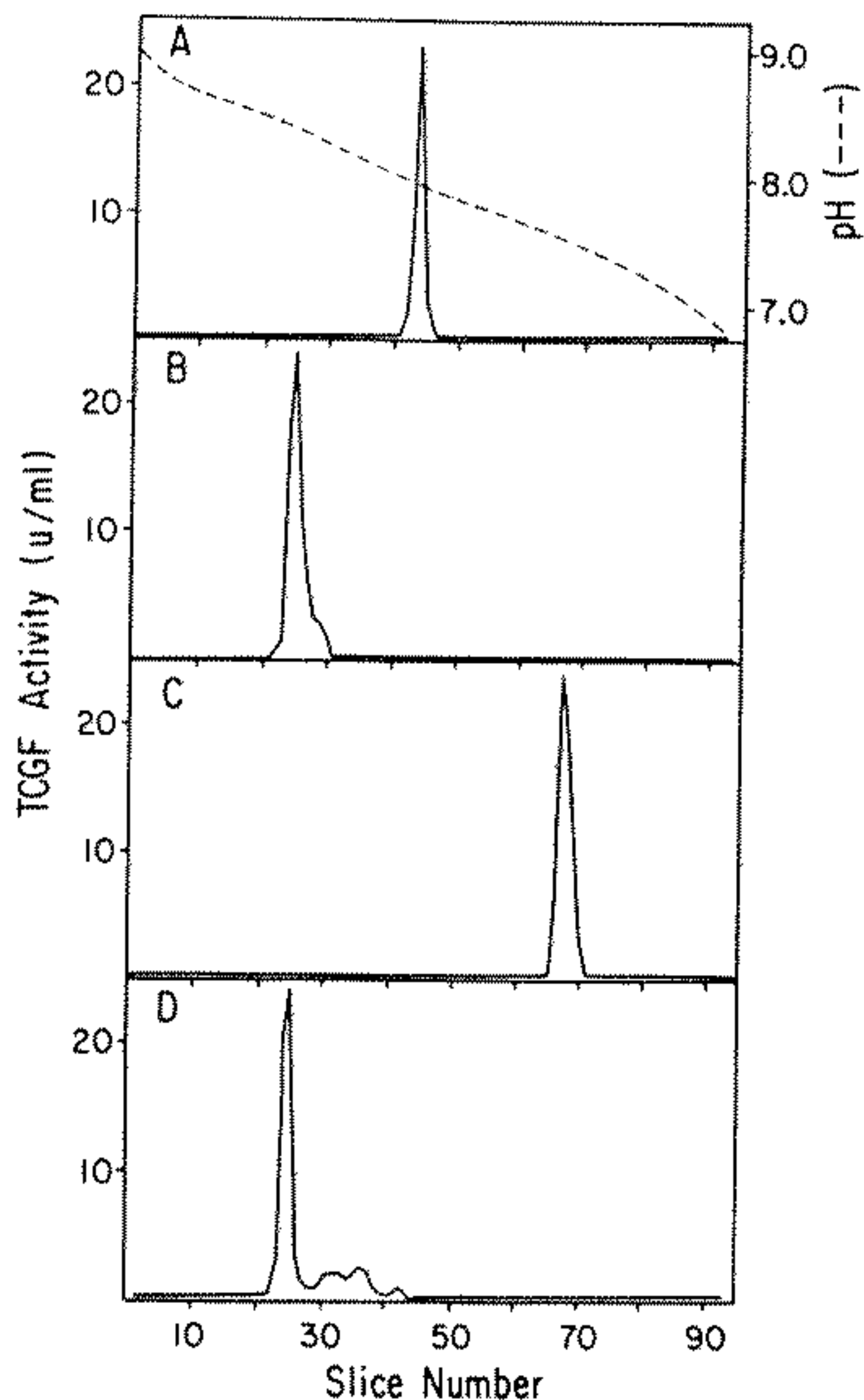


Fig. 2. IEF gels (Pharmolyte, pH 6.5–9). (A) Peak b isolated from the original IEF gel (Fig. 1A). (B) Peak b after incubation with neuraminidase. (C) Peak c isolated from the original IEF gel. (D) Peak c after incubation with neuraminidase. The pH gradient illustrated in A was determined on a parallel gel.

Inhibition of glycosylation

As an alternative approach to examine the presence of carbohydrate, TCGF was prepared from tonsil cells in the presence of 2-deoxy-D-glucose. At a concentration of 7 mM 2-deoxy-D-glucose, only 35% of the normal level of TCGF was released. The residual activity, however, was significantly enriched in the high-pI, asialo form of TCGF as detected by IEF. As shown in Fig. 3A, the control culture yielded TCGF that was evenly distributed into three IEF peaks, whereas for the 2-deoxy-D-glucose culture (Fig. 3B), the majority of the activity focused at the higher isoelectric point.

Heterogeneity on SDS-PAGE

The heterogeneity of TCGF prepared from tonsil cells was also demonstrable on SDS-PAGE. As shown in Fig. 4A, TCGF isolated by gel chromatography separated into two peaks of activity after SDS-PAGE with

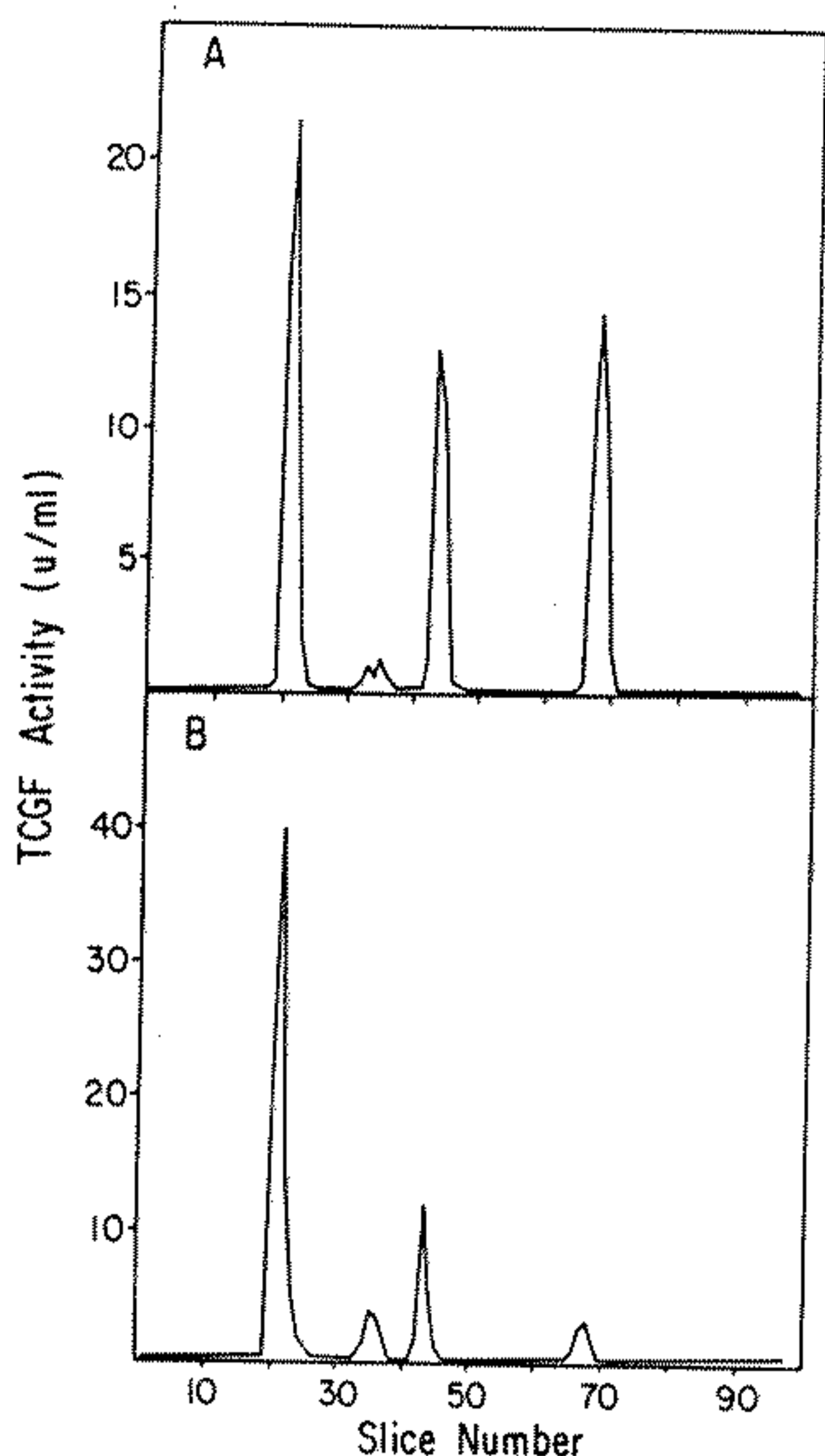


Fig. 3. IEF gels (Pharmolyte, pH 6.5–9) of TCGF prepared from a single tonsil in the absence (A) and presence (B) of 7 mM 2-deoxy-D-glucose (120 units TCGF loaded on each gel).

nominal molecular sizes (M_r) of 16,200 and 14,600. Essentially the same results were obtained with and without 2-mercaptoethanol in the sample buffer. This apparent heterogeneity in M_r appeared to be related to variable glycosylation and sialylation, since the activity in each of the three peaks isolated by IEF migrated as single entities on SDS-PAGE. The R_f value of IEF peak a activity corresponded to that of the low- M_r peak, whereas the R_f values of IEF peaks b and c activity corresponded to that of a high- M_r peak shown in Fig. 4A. Furthermore, electrophoresis of TCGF from the JURKAT cells, which did not appear sialylated on the basis of a lack of shift in pI after treatment with neuraminidase, gave rise to a single peak of activity with an R_f value close to that of the low- M_r peak of the tonsil material (Fig. 4B). To directly test the influence of sialic acid residues on SDS-PAGE, the tonsil material isolated by gel chromatography was treated with neuraminidase prior to electrophoresis: this resulted in conversion of the activity to a closely spaced doublet with R_f values similar to that of

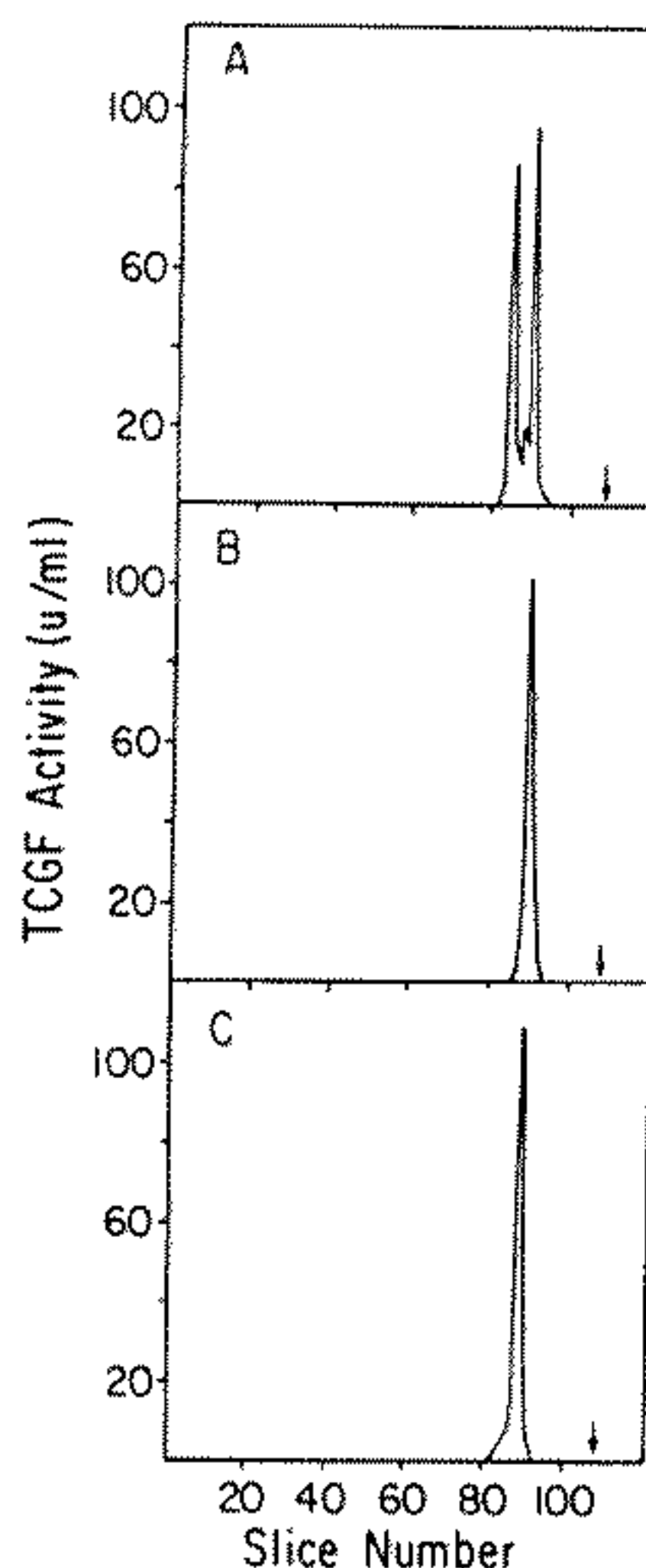


Fig. 4. SDS-PAGE of TCGF preparations. (A) Sephadex G100 pool of tonsil-derived TCGF. (B) Sephadex G100 pool of JURKAT cell line-derived TCGF. (C) Sephadex G100 pool (tonsil) after incubation with a mixture of glycosidases and neuraminidase (*D. pneumoniae*). The arrows indicate the position of the tracking dye. Approximate mol. wts were determined with a parallel gel using the following standards: bovine serum albumin, ovalbumin, trypsinogen, myoglobin and lysozyme.

the original low- M_r peak. Incubation with a mixture of endo- and exoglycosidases and neuraminidase (*D. Pneumoniae*) converted all of the activity to a single peak (Fig. 4C). Thus, the detectable differences in migration on SDS-PAGE for TCGF prepared from tonsils were due primarily to a variable sialic acid content and, to a lesser extent, variations in other sugar residues.

Maintenance of T-cell proliferation

To test whether TCGF activity (determined by the TCGF microassay) which migrated as single peaks on IEF and SDS-PAGE would support the continuous proliferation of T-cells, a cloned murine cytotoxic T-cell line (CTLL-2, subclone 15H) was cultured in material isolated as peaks a–c from IEF, and in material treated with neuraminidase and glycosidases prior to isolation from SDS-PAGE. As shown in Fig. 5, each of these TCGF preparations supported the continuous growth of this T-cell line over successive passages. The fact that asialo-TCGF

Table 1. TCGF activity: murine vs human target cells

Sample	Mouse T-cell line		Human T-cell blasts	
	50% endpoint ^a	Units/ml	50% endpoint	Units/ml
TCGF standard	10.6	(1)	12.1	(1)
IEF ^b peak a	6390	603	7020	580
IEF peak b	3920	370	4300	355
IEF peak c	6040	570	6780	560
SDS-PAGE ^c peak	1240	117	1270	105

^aThe 50% endpoint is the reciprocal of the sample dilution giving 50% of the maximal incorporation of [³H]Tdr in the TCGF microassay.

^bIEF peaks a-c were prepared from tonsil-derived material as in Fig. 1A.

^cThe SDS-PAGE peak was prepared from neuraminidase and glycosidase-treated tonsilar TCGF as in Fig. 4C.

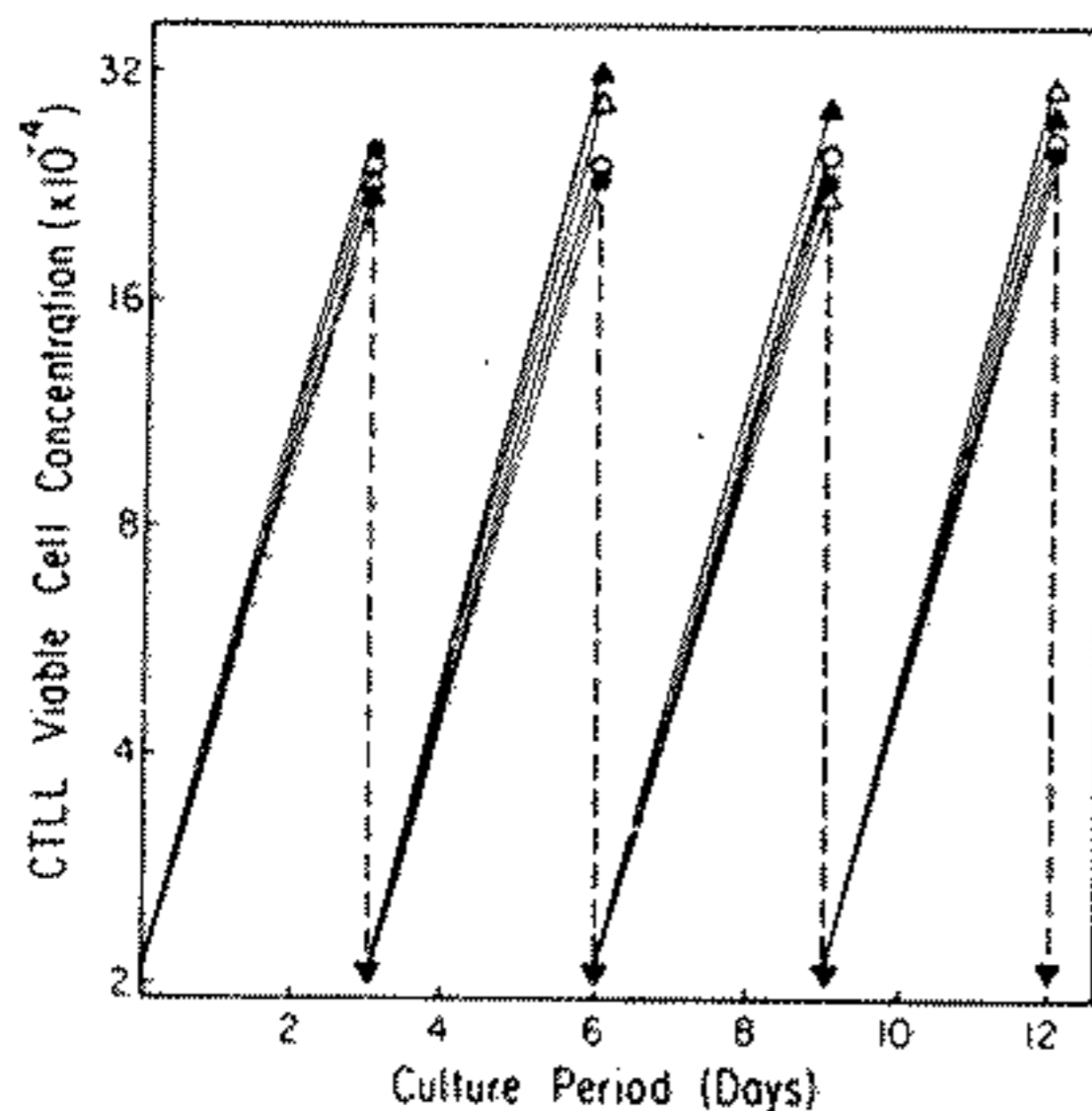


Fig. 5. Growth of CTLL subclone 15H in media containing tonsil-derived TCGF (2 units/ml) isolated from: IEF peak a (●); IEF peak b (○); IEF peak c (▲); neuraminidase and glycosidase-treated TCGF isolated from SDS-PAGE (△).

(IEF peak a and the SDS-PAGE fractions) supported the growth of the cells as well as the sialylated TCGF moieties (IEF peaks b and c) thus indicates that sialylation is not required for *in vitro* biological activity.

To rule out differences in interspecies responses, the ability of TCGF to support activated human T-cells as well as the mouse cytotoxic T-cell line was directly compared using the TCGF microassay (see Materials and Methods). As is evident in Table 1, the activity of asialo and sialylated TCGF from the various fractions was comparable on both cell types.

DISCUSSION

The results of this study indicate that TCGF activity derived from normal human tonsil cells can be ascribed to a variably glycosylated and sialylated molecule. This conclusion is based on the results of several experimental approaches: treatment with neuraminidase reduced the heterogeneity of TCGF activity on IEF to a

single, more basic isoelectric point; inclusion of an inhibitor of glycosylation during stimulation of the producing cells resulted in a shift of activity to the same isoelectric point; and digestion with glycosidases and neuraminidase eliminated the apparent size heterogeneity of TCGF activity on SDS-PAGE. While differences in amino acid sequence, particularly in neutral residues, or small differences in size (<500 daltons), may have gone undetected with the present methodology, the results are consistent with variable glycosylation as the principal cause of the heterogeneity of TCGF. In addition, the similarity in electrophoretic behavior between JURKAT-derived TCGF and glycosidase-treated tonsilar TCGF suggests that the activity released by both sources is due to the same basic molecule.

A previous study (Mier & Gallo, 1980) suggested that human TCGF is not glycosylated on the basis of a lack of binding of the activity to lectin-coupled affinity columns. We have also found little binding of tonsil-derived TCGF activity isolated from the three IEF peaks to any of several lectin-coupled columns.* If sialic acids were removed from IEF peaks b and c, however, specific binding was demonstrable to *Ricinus communis* agglutinin I affinity columns.† In addition, significant binding of JURKAT-derived TCGF to soybean agglutinin was found, indicating that while it lacked sialic acids, at least a portion of JURKAT-derived TCGF was glycosylated. The number and size of the carbohydrate chain(s) and their form of linkage to the polypeptide remain to be determined. In this regard, 2-deoxy-D-glucose, which interferes with N-linked glycosylation through its effect on

*The following lectins were examined: soybean agglutinin, wheat germ agglutinin, *Lens culinaris* agglutinin, peanut agglutinin, and *Ricinus communis* agglutinin I and II.

†Robb & Smith, unpublished observations.

the lipid-linked oligosaccharide intermediate (Datema & Schwarz, 1978), promoted a shift in the IEF pattern to the more basic, asialo form of TCGF. Assuming a direct effect of 2-deoxy-D-glucose on TCGF biosynthesis, this result suggests that the carbohydrate chain(s) containing the sialic acids is attached to the polypeptide via asparagine.

The similarity in the migration of TCGF activity on SDS-PAGE in the presence and absence of a reducing agent indicates that the molecule is not composed of disulfide-linked subunits. Our most recent observations derived from gel electrophoresis of highly-purified, radiolabelled TCGF prepared from JURKAT cells internally labelled with [³⁵S]methionine or [³⁵S]cysteine support this impression.* Although the estimated sizes of TCGF from SDS-PAGE (14,000–16,000 Mr) and gel filtration (19,000–20,000 Mr) are close enough to be consistent with a monomeric structure in the native state, further examination of the hydrodynamic behavior of the molecule is required to rule out a noncovalent, dimeric structure.

Previous studies by ourselves (Smith, 1980) and others (Larsson *et al.*, 1980; Mier & Gallo, 1980) suggested that TCGF supplied the mitogenic signal to activated T-cells rather than antigen or lectin. Since the mitogenic activity could not be ascribed to a single molecular entity, however, it remained possible that T-cell proliferation was mediated by the interaction of several soluble factors. Thus, while TCGF has yet to be purified to homogeneity, the observation that the growth of cloned cytolytic T-cells was maintained by glycosidase-treated TCGF that was relatively uniform with respect to size and charge, suggests that T-cell proliferation is mediated by a single, variably-glycosylated polypeptide and not a class of unrelated molecules. Since TCGF is obligatory for the clonal expansion of effector T-cells (Smith, 1980; Larsson *et al.*, 1980) and appears to interact with activated T-cells by means of specific binding sites (Coutinho *et al.*, 1979; Bonnard *et al.*, 1979; Smith *et al.*, 1979), by analogy to polypeptide hormone systems, we may anticipate that alterations of TCGF structure or TCGF-cell binding could lead to disease states manifested either by hypo- or hyperimmune responsiveness.

The observation that TCGF is glycosylated

and in particular, the fact that it appears to be variably sialylated, may be of physiologic and pharmacologic importance. The rapid removal of circulating desialylated glycoproteins is well documented, and is mediated by a carbohydrate recognition system present only in hepatocytes (Neufeld & Ashwell, 1979). The initial step in clearance involves specific binding to a plasma membrane receptor that recognizes glycoproteins having terminal galactose or glucose residues on their oligosaccharide chains. Since desialylated TCGF bound to galactose-specific Ricin I affinity columns, it appears that desialylated TCGF could be susceptible to rapid, physiologic, hepatic clearance. Thus, like erythropoietin, one might anticipate that desialylated TCGF might have *in vitro* biological activity but no *in vivo* biological activity (Goldwasser *et al.*, 1974). From the potential immunotherapeutic standpoint, the removal of all of the carbohydrate component may improve the *in vivo* half-life of the molecule, as has been demonstrated for interferon (Bose & Hickman, 1977), and thus prolong biological activity, since the carbohydrate component does not appear to be required for the mediation of T-cell proliferation.

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