Characterization of Carcinoembryonic Antigen Fractionated by Concanavalin A Chromatography

Henry S. Slayter and John E. Coligan

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SUMMARY

A small percentage (15%) of particles morphologically distinct from carcinoembryonic antigen (CEA) cruller-like particles (averaging 9 x 40 nm) as determined by electron microscopy, and previously presumed to be impurities, has been removed from CEA preparations by concanavalin A (Con A) affinity chromatography. The first of four affinity fractions constituted 45% of the recovered weight; the last, which constituted 17% of the recovered weight, was obtained only after a 2-day soak in 20% α-D-mannopyranoside. The affinity fractions were of essentially equivalent specific activity, morphological appearance, and composition. They all demonstrated markedly different chemical compositions and an approximately 100-fold higher specific activity than the bulk of the nonaffinity material. Thus, the bulk of the Con A-nonbound fractions apparently is not CEA but contaminating particles of several different varieties.

Compositional analyses further indicated that the mannose content of various CEA fractions was directly correlated with Con A binding affinity, amino acid content, morphological type, and CEA specific activity. This led to the conclusion that the protein chain of the various CEA preparations fractionated by us is characteristic.

An attempt to fractionate very impure crude CEA by Con A affinity chromatography indicated that this scheme cannot by itself produce as pure CEA as standard methods.

Thus, for the first time the effectiveness of the purification of a glycoprotein has been successfully monitored by electron microscopy, demonstrating that the cruller-like appearance of the CEA particle is closely related to antigenic specificity.

Whether antisera to our affinity fractions will improve the specificity and sensitivity of the clinical assay is currently being investigated.

INTRODUCTION

CEA is a tumor-associated glycoprotein that was initially described by Gold and Freedman (20, 21). Slayter and Coligan (33) demonstrated that CEA consists largely of relatively homogeneous, morphologically distinctive cruller-like particles with dimensions of 9 x 40 nm. For the purpose of further discussion, we will refer to such particles as CEA particles.) The NHE-terminal amino acid sequence of 25 residues is remarkably constant from one preparation to the next (7, 10, 36), and the chemical composition of CEA appears to be characteristic of the molecule (37).

However, CEA does possess some polymorphic properties. It exhibits charge heterogeneity when examined by isoelectric focusing (4, 11, 39) related in part to variation in sialic acid content. The multiple fractions are essentially antigenically equivalent (11).

Recently, Rogers et al. (31) have shown that CEA can be separated into 2 fractions (bound and unbound) by chromatography on columns of Con A bound to Sepharose. In addition to sharing common antigenic determinants, these 2 fractions possessed immunologically distinguishable antigenic specificities. The immunological distinction between Con A-bound and unbound CEA raises questions concerning the chemical origins of this difference.

In this paper we report the chemical, immunological, and electron microscopic characterization of fractions obtained by chromatography of CEA on Con A columns.

The occurrence in CEA preparations of populations of molecules with different chemical properties and antigenic specificities may account, in part, for the lack of diagnostic specificity observed in currently available radioimmunoassays (27, 37). It is hoped that a more chemically homogeneous preparation of CEA may alleviate this problem.

MATERIALS AND METHODS

Preparation of CEA. CEA used for Con A fractionation was purified by established procedures (12, 26, 30, 38). In brief, liver metastases of colon cancer were homogenized and mixed with an equal volume of 2 M perchloric acid. The supernatant obtained after centrifugation, and dialysis was chromatographed on Sepharose 4B. Fractions containing CEA activity were pooled and rechromatographed on Sephadex G-200. CEA from individual tumors was never pooled.

While the extended use of perchloric acid treatment as a means of glycoprotein extraction can cause denaturation, our experience has been that the amount of CEA antigenic activity present in the supernatant after perchloric acid precipitation equals that amount that was present in the tumor homogenate, prior to the addition of perchloric acid. However, if the supernatant is not neutralized relatively soon...
after the removal of the precipitate, up to 50% of the antigenic activity can be lost (unpublished results). This loss is attributed to the previously reported acid lability of CEA (13). CEA purified without the use of perchloric acid precipitation is very similar if not identical in its physical, chemical, and antigenic properties to the CEA described here.

Radioimmune Assay for CEA. CEA was measured by double antibody radioimmune assay using antiserum to CEA that had been prepared as described above (16, 18).

Amino Acid Analysis. Amino acid analyses were performed on CEA samples that had been hydrolyzed with p-toluenesulfonic acid in duplicate for 24 and 72 hr by a modification (13) of the method of Liu and Chang (28). A Beckman Model 121H amino acid analyzer was used.

Carbohydrate Analysis. Values for neutral sugars were obtained by the gas chromatographic procedure of Clamp et al. (9). Amino sugars were determined on the amino acid analyzer. Sialic acid was measured by the method of Warren (42).

Electron Microscopy. Electron microscopy was performed as described previously (33).5

Fractionation of CEA by Con A-Sepharose Affinity Chromatography. Affinity chromatography was based on the procedure of Rogers et al. (31), except that the final step included a 2-day soak in 20% methyl α-D-mannopyranoside prior to elution with the same sugar derivative (see Chart 1 for details). This latter step accounted for the elution of a high-affinity fraction not observed by these investigators. Fractionated material was dialyzed extensively versus water until sugar was no longer detectable in the dialysate by the phenol sulfuric method (15). Dialysis versus water was continued for 2 or 3 more days. The samples were lyophilized and dried in a desiccator over silica gel or P2O5, before being weighed for analysis.

RESULTS

Con A Affinity Chromatography of CEA. In this study, CEA purified as previously described (12, 30, 38) was further fractionated on a Con A column (Chart 1). Three nonaffinity peaks (Fractions I, II, and III) [The difference in their elution volume is thought to be due to differences in particle size (see Fig. 2.)] and 4 affinity fractions (Fractions V to VIII) were consistently reproduced during repetitive runs.

Fraction V material comprised about 45% of the total dry weight recovered (Table 1). The other main affinity fraction (VIII), which was obtained after soaking and elution with 20% methyl α-D-mannopyranoside, comprised 17% of the recovered weight. Approximately 90% of the recovered material was specifically attached to the Con A column (Fractions V to VIII).

The total weight recovery is considered good in view of the number of manipulations, such as a lengthy dialysis.

Relative Specific Activities of the CEA Con A Fractions. The specific activities relative to the CEA prior to Con A fractionation are shown in Table 1. The specific activity of the material eluted in the affinity fractions (V to VIII) is slightly increased compared to the starting material. The specific activities of the nonaffinity fractions (I to IV) are considerably lower than those of the affinity fractions. A significant portion of the CEA activity present in the nonaffinity fractions is probably due to leakage of high-specific activity CEA from the Con A column (discussed below). All the Con A fractions, if added in sufficient amount, were capable of complete inhibition in the radioimmune assay.

Electron Microscopic Appearance of the CEA Con A Fractions. CEA prior to Con A chromatography contains a small proportion (about 15%) of particles of a morphological type that is different from the typical cruller-like CEA particle (Fig. 1a). These particles range from long rods to short rods and nearly spherical particles. Electron micrographs indicate that these irregularly shaped particles are separated from the more typical CEA particles by Con A chromatography.

Fig. 2 shows the appearance of Con A Fractions I, II, and III. In general, Fraction I is composed of long rods approximately 3 to 4 nm wide and varying in length up to 100 nm. Fraction II consists of a mixture of these long rods with some CEA particles, and some essentially spherical particles, along with some shorter rods approximately 4 x 50 nm. Fraction III, on the other hand, consists of a mixture of CEA particles, practically no long rods, a significant number of small spherical particles approximately 10 nm in diameter, and short thin rods 20 to 70 nm long and about 1.5 nm wide. Fig. 3 shows the cruller-like homogeneous appearance of Fractions V, VI, and VIII. Chart 2 indicates that Fraction V has a peak length of about 43 nm. Fraction VII is indistinguishable from the other affinity fractions in both morphological appearance and chemical composition.

Chemical Composition of the CEA Con A Fractions. Differences occur in the carbohydrate compositions of the various Con A fractions (Table 2). As expected, due to the nature of Con A binding specificity, all nonaffinity fractions...
Table 1
Relative specific activities and percentage of the total weight recovered for the Con A fractions obtained from the fractionation of CEA and ascending Pool A

<table>
<thead>
<tr>
<th>CEA</th>
<th>Ascending Pool A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative specific activity</td>
<td>% of total wt recovered</td>
</tr>
<tr>
<td>Prior to Con A fractionation</td>
<td>1.00</td>
</tr>
<tr>
<td>Fraction I</td>
<td>0.05</td>
</tr>
<tr>
<td>Fraction II</td>
<td>0.02</td>
</tr>
<tr>
<td>Fraction III</td>
<td>0.11</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>0.38</td>
</tr>
<tr>
<td>Fraction V</td>
<td>1.01</td>
</tr>
<tr>
<td>Fraction VI</td>
<td>1.06</td>
</tr>
<tr>
<td>Fraction VII</td>
<td>0.96</td>
</tr>
<tr>
<td>Fraction VIII</td>
<td>1.12</td>
</tr>
<tr>
<td>Fraction IX</td>
<td>Trace</td>
</tr>
<tr>
<td>% recovery of the total wt applied</td>
<td>89.5</td>
</tr>
</tbody>
</table>

* The specific activities are relative to the CEA prior to Con A fractionation. This pool had an actual specific activity of 0.97. The specific activities are calculated by dividing the radioimmunoassay antigenic activity by the dry weight.

** ND, not determined; trace, not weighable.

Chart 2. Histogram showing the distribution of lengths of CEA particles in the peak pool, Fraction V material.

contain small amounts of mannose. Mannose content increases with fraction number. Galactose content is increased in nonaffinity Fractions II and III compared to the affinity fractions. N-Acetylgalactosamine is significantly enriched in the nonaffinity fractions compared to the affinity fractions. Fucose and N-acetylglucosamine are present in relatively constant amounts in all the fractions. Fractions III and IV are enriched in sialic acid.

Amino acid variations among the fractions are presented in Table 2. Major differences in amino acid composition are shown between the nonaffinity and affinity fractions. Proline, threonine, alanine, histidine, serine, and glycine are increased in some or all nonaffinity fractions, whereas glutamic acid plus glutamine, isoleucine, leucine, and tyrosine are increased in the affinity fractions. Differences in cysteine and tryptophan may be significant, but they could also be due to the fact that in some cases only small amounts of material were available for analysis.

No significant differences are evident among the affinity fractions.

The percentages of carbohydrate and protein in the Con A fractions recovered are also shown in Table 2. Fractions II and III are significantly more enriched in carbohydrate than are the affinity fractions.

The percentage of total weight recovery (carbohydrate plus protein) after analysis was 75 to 85% for most fractions, which agrees with previous compositional studies on CEA (33, 37). The failure to achieve 100% recovery is attributed to the rapid uptake of H₂O by CEA during weighing prior to aliquoting for compositional analysis.

**Relationship of Mannose Content to CEA Antigenic Activity and Protein Content.** Chart 3 illustrates the approximately linear correlation of mannose content with CEA antigenic activity. The cluster of points at the top of the plot (solid line) probably signifies that CEA specific activity maximizes in this purification and assay system at about 9% mannose, which corresponds to about 50% protein.

No other sugar except N-acetylgalactosamine, which decreases 3-fold, shows as marked a change as mannose does during purification.

There is also a direct correlation between mannose and protein content (Chart 3, dashed line). This relationship becomes apparent only after preliminary purification on Sepharose 4B and appears to approach a maximum value of 50% protein asymptotically.

**Refractionation of Nonaffinity Fractions by Con A Affinity Chromatography.** The question of whether the CEA activity present in the nonaffinity fractions was inherent or was due to a small amount of leakage from the affinity fractions was examined. Fractions I, II, and III (1.4, 3.4, and 0.9 mg of dry weight, respectively, from a Con A fractionation similar to that shown in Chart 1) were recombined and refractionated on the Con A column. In addition to the usual nonaffinity fractions, a fraction equivalent in position to Fraction V of previous fractionations was obtained. The specific activities of the recovered Fractions I and II were both reduced by...
Table 2

| Chemical composition of Con A fractions obtained from the fractionation of CEA |
|-----------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                  | Unfractionated  | Fraction I       | Fraction II      | Fraction III     | Fraction IV     | Fraction V       |
|                                  | moles carbohydrate/10^5 g carbohydrate | moles carbohydrate/10^5 g carbohydrate | moles carbohydrate/10^5 g carbohydrate | moles carbohydrate/10^5 g carbohydrate | moles carbohydrate/10^5 g carbohydrate | moles carbohydrate/10^5 g carbohydrate |
|                                  | Fucose^a        | Mannose          | Galactose        | N-Acetylgalactosamine^c | N-Acetylgalactosamine^c | N-Acetylgalactosamine^c |
|                                  | 104.4           | 79.6            | 108.3            | 231.0            | 12.5            | 16.0            |
|                                  | 96.3            | 2.0             | 183.6            | 190.7            | 65.8            | 12.5            |
|                                  | 114.5           | 13.0            | 182.8            | 175.0            | 43.4            | 22.3            |
|                                  | 123.1           | 47.7            | 88.5             | 208.5            | 35.9            | 23.6            |
|                                  | 114.0           | 91.7            | 123.5            | 209.5            | 8.3             | 13.5            |
|                                  | 110.3           | 97.9            | 114.9            | 219.6            | 4.0             | 13.2            |
|                                  | 105.2           | 108.0           | 120.1            | 204.1            | 9.9             | 13.9            |
|                                  | moles amino acid/10^5 g protein | moles amino acid/10^5 g protein | moles amino acid/10^5 g protein | moles amino acid/10^5 g protein | moles amino acid/10^5 g protein | moles amino acid/10^5 g protein |
|                                  | Lysine^e        | Histidine        | Arginine         | Aspartic acid or asparagine | Threonine        | Serine           |
|                                  | 25.7            | 24.2            | 23.3             | 134.3            | 81.8            | 98.4            |
|                                  | 18.4            | 57.7            | 29.0             | 91.2             | 141.1           | 113.9           |
|                                  | 21.0            | 31.6            | 29.5             | 122.4            | 70.2            | 130.7           |
|                                  | 22.9            | 46.6            | 46.0             | 85.6             | 79.5            | 93.8            |
|                                  | 22.1            | 21.8            | 21.8             | 80.4             | 53.8            | 97.9            |
|                                  | 19.5            | 23.2            | 23.2             | 71.8             | 100.1           | 84.5            |
|                                  | 24.8            | 21.4            | 21.4             | 75.7             | 95.6            | 88.7            |
|                                  | % of the wt recovered | % of the wt recovered | % of the wt recovered | % of the wt recovered | % of the wt recovered | % of the wt recovered |
|                                  | Carbohydrate    | 51.0            | 77.7            | 67.0             | 54.6            | 49.9            |
|                                  | 49.0            | 22.3            | 33.0            | 45.4             | 50.1            | 52.0            |
|                                  | Protein         | 1699            | 1699            | 1699             | 1699            | 1699            |

Con A Affinity Chromatography Will Not Readily Purify Crude CEA. Several investigators (5, 6, 8, 14) have suggested the possibility that Con A affinity chromatography might offer an alternative to perchloric acid treatment and/or molecular sieving for the purification of CEA. Therefore, the capability of Con A affinity chromatography to purify CEA from a highly impure CEA preparation was examined.

The ascending Pool A from the Sephadex G-200 column shown in Chart 4 was chosen for this experiment. The specific activity of Pool A (Table 1) was very low (0.12 mg CEA activity per mg dry weight). This material had been purified to this stage by established methods (12, 30, 38). However, the absorbance profile (280 nm) versus the CEA...
Con A affinity chromatography of Pool A resulted in an elution profile similar to that shown in Fig. 1, and similar Pool A was present in the affinity fractions (V to VIII) compared to 90% in the previously fractionated CEA (Table 1).

The specific activities of Pool A affinity fractions were enhanced approximately 3-fold relative to the starting material (Table 1). However, these specific activities were significantly lower than in the equivalent affinity fractions obtained from the fractionation of high-quality CEA. The non-affinity fractions (I, II, and III) had specific activities nearly identical to those of the equivalent non-affinity fractions obtained from high-quality CEA.

DISCUSSION

The purification of CEA has been based upon extraction of homogenates of tumor tissue in perchloric acid followed by Sepharose 4B and Sephadex G-200 chromatography (37). Krupay et al. (26) use an additional zone electrophoresis step which may or may not alter the product (37). Electron microscopic studies conducted in this and a previous study indicated that CEA preparations are relatively homogeneous, consisting mainly (85%) of unique cruller-shaped molecules (average dimensions, 40 x 9 nm). It remained to be determined whether the irregularly shaped particles (approximately 15%) were alternate forms of CEA or impurities.

Fractionation of CEA on a Con A affinity column produced 9 fractions, 4 nonaffinity fractions and 5 affinity fractions. Two of the latter fractions (V and VIII) contained the bulk of the CEA of high specific activity. The 2nd major affinity fraction was obtained by soaking the column in 20% methyl α-D-mannopyranoside for an extended period (usually 48 hr), prior to final elution. Elution with 20% methyl α-D-mannopyranoside immediately after the 10% methyl α-D-glucopyranoside elution did not cause significant elution of additional material even after about 2 column bed volumes had passed. This latter observation probably explains why Rogers et al. (31) failed to recover any material in their 20% methyl mannoside wash. Consequently, their recovery based on absorbance applied and eluted was only 80%. Boenisch and Norgaard-Pedersen (5) were able only to recover 50% of the CEA activity applied to a Con A column using 1.9% methyl α-D-glucopyranoside to elute the column. No physical or chemical studies were performed on the fractions produced in the above investigations.

When Con A-fractionated CEA was analyzed by electron microscopy, the percentage of characteristic CEA particles increased almost 100% in the affinity fractions compared to 85% in the precolumn material. In addition, the specific activities of the affinity fractions were increased...
slightly relative to the starting material.

Since the CEA specific antiserum used in the radioimmunoassay were raised to CEA preparations equivalent to the starting material used for our Con A fractionation, and since only about 15% by weight of morphologically distinct impurities were removed by affinity chromatography, the specific activity of the purified fractions would not be expected to increase more than the small amounts observed.

It seems likely that the difference in Con A binding affinity between the bound fractions can be attributed to quantitative or qualitative differences in the mannose content, which are not unexpected in view of known microheterogeneity of glycoproteins (35). Con A is known to bind specifically to terminal α-linked mannose and internal α-1,2-linked β-mannose residues (22, 23), both of which are present in CEA (13, 24). These residues account for approximately 25% of the total mannose residues in CEA (24). Recent experiments have shown that Con A Fraction VIII contains more α-1,2-linked mannose than does Fraction V (J. E. Coligan and D. G. Pritchard, unpublished results), whereas the amount of terminal mannose was similar in both fractions. Con A also binds to terminal α-linked N-acetyl-D-glucosamine (22). Periodate oxidation studies (13, 17) and methylation analysis (24) indicated that little of this residue is present in most CEA preparations (13). Also recent results (J. E. Coligan and D. G. Pritchard, unpublished results) indicate no increase in this residue in Fraction VIII versus Fraction V.

Approximately 20% of the CEA applied to the Con A affinity column by Rogers et al. (31) was recovered as a peak with a 10% methyl α-D-glucopyranoside wash and contained a significant amount of nonspecific cross-reacting antigen (NCA, also known as NGP and CCEA-2) (see Refs. 29, 37, and 40).

The use of gel columns in our method of purification probably eliminates this low-molecular-weight material (38). This is supported by the failure of antiserum to NGP or CCEA-2 to react with the Con A fractions, by immunodiffusion.

The nonaffinity fractions were very low in CEA activity and contained a variety of morphological types. Fraction I contained 3-nm-wide rods of variable length up to 200 nm, reminiscent of certain cell surface glycoproteins (32) and of blood group substances (34). The antigenic determinant of Blood Group A substance is N-acetylgalactosamine, a constituent that was increased in amount in the nonaffinity fractions. Several investigators (19, 39) have reported Blood Group A activity in CEA preparations as well as other blood group determinants (25), but Blood Group Substance A does not appreciably inhibit in the CEA radioimmunoassay (37). In this study, Fractions I, II, and III were not reactive to antiserum against A, as well as B, AB, or D typing sera in double diffusion experiments (H. S. Slayter, unpublished work).

The approximately 10% of CEA eluted in Fractions I, II, and III correlates with the 15% of the particles that did not conform to the CEA morphology before Con A fractionation. The slight tendency for the percentage by weight to be on the low side of the particle count figure can be accounted for on the basis that most of the non-CEA particles are probably of lower molecular weight based on size and shape.

Refractionation of pooled Fractions I, II, and III reduced by only 40% the specific activity in Fractions I and II. It would thus appear that the residual CEA activity in Fractions I, II, and III is due only partially to leakage, with a significant amount being due to cross-reactive or antigenic determinants identical to those present in the affinity fractions. However, since CEA antigenic determinants are known not to involve mannose residues (discussed below), it is possible that the residual activity is due to CEA deficient in terminal and 1,2-linked mannose residues. It is unlikely that overloading of the Con A column is responsible for leakage since the percentage of CEA activity in the nonaffinity fractions did not increase with larger column loads.

It has been suggested that Con A fractionation might provide an alternative method or substitute for steps in the current purification scheme of CEA. Our data do not support this proposal. When largely impure CEA material (Pool A) was fractionated on a Con A column, the affinity fractions were somewhat enriched, but still low, in specific activity. Furthermore, these retain a significant number of morphological types considered to be atypical of CEA. Thus, it seems unlikely that very crude CEA starting material would yield pure CEA by a single passage over a Con A affinity column.

The chemical nature of the antigenic determinant(s) of CEA is currently a controversial topic. Studies by Banjo et al. (1, 3) have suggested the N-acetylgalactosamine residues in the inner core of the polysaccharide chains play a central role in the immunodominant groupings. The linkage between asparagine and N-acetylgalactosamine has also been implicated in the tumor-specific site (2). Other studies (13, 17, 24, 41) have suggested that the protein portion of CEA contains the immunodominant site(s). Differences in antisera could be an explanation for these contradictory results, but this seems unlikely (24).

All the above investigators, however, do agree that sialic acid, fucose, galactose, and mannose are not involved in the antigenic specificity of CEA. If the protein portion of CEA contains the antigenic determinants, then the observed linear relationship between mannose content and specific activity in this study may be explained by the linear relationship between mannose and protein content.

The observation that the Con A affinity fractions are essentially identical in total protein, amino acid content, and specific activity, although mannose content increases slightly, suggests that for the various CEA preparations fractionated by us the protein chain of the affinity fractions is characteristic. This will have to be verified by amino acid sequence analysis.

Con A fractionation of high-quality CEA results in nonaffinity fractions that contain essentially all of the non-CEA shaped particles, whereas the affinity fractions contain essentially 100% characteristic CEA particles. It appears that the cruller-like appearance of the CEA particle is a characteristic closely related to CEA antigenic specificity and chemical composition. This relationship has been found to be invariant in a number of tumors fractionated and analyzed in this way.
Thus, for the 1st time the effectiveness of the purification of a glycoprotein has been successfully monitored on a morphological basis by electron microscopy. Whether antisera to these affinity fractions will improve the specificity and sensitivity of the clinical assay is currently being investigated.

ACKNOWLEDGMENTS

This work was performed with excellent technical assistance of Nancy Grove, Vickie Vanik, and Emmanuel Ojo. Amino acid determinations were performed by Dr. Marianne Egan and Laurence Wimmer. We also wish to acknowledge the assistance of William Winchell and Joseph Rothchild in porting this work. We wish to thank Dr. Charles Todd for encouragement and support during the course of this work. Antiserum to NGP and CCEA-2 were kindly provided by Dr. J-P. Mach and Dr. D. A. Darcy, respectively.

REFERENCES

Fig. 1. a, electron micrograph of CEA before Con A fractionation. Arrowhead, CEA morphological type. Rotary shadow-cast with platinum. Bar, 0.1 μm. b, G-200 ascending Pool A before Con A fractionation. c, G-200 ascending Pool A Fraction V after Con A fractionation. Rotary shadow-cast with platinum. Bar, 0.1 μm. x 100,000.

Fig. 2. Electron micrographs of low-specific-activity Con A fractions of CEA. a, Fraction I; b, Fraction II; c, Fraction III. Rotary shadow-cast with platinum. Bar, 0.1 μm. x 100,000.
Fig. 3. Electron micrographs of high-specific-activity fractions of CEA. a, Fraction V; b, Fraction VI; c, Fraction VIII. Rotary shadow-cast with platinum. Bar, 0.1 μm. × 100,000.
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