AFFINITY PURIFICATION OF β-ENDORPHIN-LIKE MATERIAL FROM NG108CC15 HYBRID CELLS BY MEANS OF THE MONOCLONAL β-ENDORPHIN ANTIBODY 3-E7

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Abstract—Neuroblastoma × glioma hybrid cells (NG108CC15) were examined for the presence of β-endorphin-like material. In order to differentiate this β-endorphin-like material from crude cell extract, a procedure for immunoaffinity chromatography was developed. The monoclonal antibody 3-E7 employed possesses the unique property of recognizing the *N*-terminal sequence of virtually all endogenous opioid peptides, but not their precursors. By means of this immunoaffinity procedure about 90% of exogenous β-endorphin was recovered from 10 ml phosphate buffered saline samples. Affinity chromatography served as first-step purification of crude NG108CC15 cell extract for the separation and concentration of β-endorphin-like material. The eluate of the immunoaffinity gel was subjected either to Sephadex gel filtration or to high pressure liquid chromatography. Under either condition, immunoreactive β-endorphin which eluted with synthetic β-endorphin was detected. The concentration in six different batches varied from 4 to 17 fmol/10^8 cells. This would be 10–200-fold lower than that observed for the enkephalins or dynorphin A/α-neo-endorphin. It is concluded that the utilization of the monoclonal antibody 3-E7 for a first-step purification of cell extracts was an essential pre-requisite for the separation of β-endorphin-like material from the hybrid cells. The presence of enkephalin-like material, of dynorphin A/α-neo-endorphin-like material and of β-endorphin immunoreactive material suggests that NG108CC15 cells are able to generate opioid peptides related to the precursors pre-proenkephalin A, pre-proenkephalin B and pro-opiomelanocortin.

The neuroblastoma-glioma hybrid cell line NG108CC15 (i.e. NG108-15, Hamprecht, 1977) has been widely employed for the study of acute and chronic actions of opioids on various neuronal functions (for reviews see Hamprecht, 1978; Gilbert and Richelson, 1983). However, these hybrid cells not only respond to opioids but also synthesize opioid peptides themselves. Those peptides reported, as yet, to exist therin comprise met- and leu-enkephalin (Glaser *et al.*, 1980, 1982; Braas *et al.*, 1983), dynorphin A and α-neoendorphin (Schulz *et al.*, 1983). The occurrence of those opioid peptides is of significance in light of the fact that they originate from distinctive precursors, that is, pre-proenkephalin A and pre-proenkephalin B (Noda *et al.*, 1982; Kakidani *et al.*, 1982).

The presence of both the enkephalins and dynor-phin A/α-neoendorphin raises speculation as to whether NG108CC15 hybrid cells might also contain β-endorphin. As yet, attempts to demonstrate the occurrence of this opioid peptide therein have failed (Braas *et al.*, 1983). On the other hand, these hybrid cells display a high density of specific binding sites for β-endorphin, at which leu-enkephalin and morphine exert a substantially lower affinity (Hammonds *et al.*, 1981). It is, thus, quite possible that NG108CC15 cells contain β-endorphin, and, thus, the gene encoding pro-opiomelanocortin (POMC, Nakanishi *et al.*, 1979).

The present paper presents evidence for the existence of β-endorphin-like material in these neuroblastom-glioma hybrid cells. An essential pre-requisite for the detection of this opioid peptide was the utilization of the monoclonal β-endorphin antibody 3-E7 (Gramsch *et al.*, 1983) linked to sepharose for the separation of the peptide from crude cell

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extracts. This affinity chromatography procedure offers a potentially powerful technique for the over-coming of many problems associated with the purification of peptides (Lowe, 1979; Pekonen et al., 1980). The virtually unlimited availability of 3-E7 antibodies in addition to their well defined character-istics (Köhler and Milstein, 1975) suggests that these antibodies may provide the possibility of an effective immunoaffinity chromatography system for the purification of opioid peptides in general.

EXPERIMENTAL PROCEDURES

Hybrid myelomas (3-E7) (Gramsch et al., 1983) secreting a monoclonal antibody to opioid peptides were cultured in RPMI1640 medium (GIBCO, Europe, Karlsruhe, F.R.G.) containing 6% fetal calf serum (Meo et al., 1983). The antibody was separated from culture supernatant by means of protein A-Sepharose (Pharmacia, Freiburg, F.R.G.) chromatography (Ey et al., 1978). The immunoglobulin eluted at pH 4.5 was pooled and immediately adjusted to pH 7.0. The concentration of the immunoglobulin was determined photometrically according to Schönenberger (1955).

Coupling of the monoclonal antibody to cyanogen bromide-activated agarose (Sepharose 4B, Pharmacia) was carried out in 0.1 M sodium citrate buffer (pH 7.0). For this purpose, the immunoglobulin concentration of the protein A eluate (pH 4.5 fraction) was adjusted with citrate buffer (0.1 M) to 1 mg/ml. Ten mg of the immunoglobulin was coupled to 1 g Sepharose 4B (12 h, 4°C) in the presence of 0.5 M NaCl. The obtained gel-ligand conjugate was washed with acetate buffer (0.1 M, pH 4), containing NaCl (0.5 M), followed by 0.1 M NaHCO$_3$ (pH 8.3). The gel was stored in 0.01 M phosphate buffer (pH 7.4) at 4°C, containing 0.1% sodium azid.

For affinity chromatography, the gel was packed in columns (0.5 × 5.0 cm) and equilibrated in phosphate-buffered saline (PBS, pH 7.4, GIBCO). If not otherwise mentioned, 10 ml of PBS containing the endorphin to be studied was applied to the column at 4°C (flow rate 0.06 ml/min). Subsequently, the column was washed with 8 ml PBS and eluted with 2.5 M acetic acid. Fractions (1 ml) of the wash and elution procedure were collected. There-after, the gel was stored in the cold in 0.01 M phosphate buffer (pH 7.4), containing 0.1% sodium azide.

Gelfiltration (column 1.0 × 90.0 cm) was performed in Sephadex G-50, superfine (Pharmacia), in 0.1 M acetic acid. Samples (0.5 ml) were applied at 4°C and a flow rate of 6 ml/h. The fractions collected (1 ml) were either neutralized and further processed or were lyophilized and taken up in an appropriate buffer for radioimmunoassay (RIA).

NG108CC15 cells were obtained from Dr B. Hamprecht (Würzburg, F.R.G.) and Dr M. Nirenberg (Bethesda, Maryland, U.S.A.). The hybrid cells employed here were obtained either from Meloy-Laboratories (Virgina, U.S.A.) or grown at the Max–Planck-Institute for Psychiatry (MPI). The Meloy-cells were harvested at the state of confluency, washed with PBS (GIBCO) and shipped on dry ice to Martinsried. The MPI-cells were cultured as described by Dawson et al. (1972) in an atmosphere of 95% O$_2$/5% CO$_2$.

At confluency the cells were harvested and washed with PBS.

For opioid peptide extraction freshly thawed (Meloy-cells) or harvested (MPI-cells) material was incubated with 0.1 N HCL at 95°C for 10 min (1 × 10^8 cells in 4 ml HCl). Thereafter the samples were homogenized (Ultra Turrax, rod N 100) for 10 s (maximal setting) and centrifuged (10,000 g, 4°C, 20 min). The clear supernatant was adjusted to pH 7.4 and to a volume of 10 ml using PBS ("crude extract").

The radioimmunoassay (RIA) procedure for β-endorphin and characteristics of the antibody employed have been described by Höllt et al. (1979). The RIA for dynorphin A and α-neoendorphin essentially followed that detailed by Maysinger et al. (1982), which also details the characteristics of the antisera employed. The tests for immunoreactivity were assayed in duplicate and the data presented represent mean values. If not otherwise mentioned, all RIA data reported are corrected for the respective tritiated peptide employed as internal standard.

High pressure liquid chromatography (HPLC, Waters, Königstein/Ts., F.R.G.) was used for an analysis of the presence of β-endorphin. The procedure followed that described by Maysinger et al. (1982). Briefly, a μ Bondapak C18 reverse phase column (3.9 × 300 mm) was eluted with a linear gradient of acetonitrile (5–40%) in 1 M acetic acid within 35 min (flow rate 2 ml/min). The recovery of β-endorphin was 90%. The fractions (1 ml) collected were lyophilized and assayed for immunoreactivity.

The separation of opioid peptides from "crude extract" was carried out by immunoaffinity chromatography, which was applied at 4°C (flow rate 0.05 ml/min). Subsequently, the gel was washed with 8 ml PBS and eluted with 2.5 M acetic acid (2 ml). The eluate was lyophilized and taken up in 750 μl buffer "D" (Guillemin et al., 1977). This material is termed "purified extract".

β-Endorphin, dynorphin A, α-neo-endorphin and leu-enkephalin were purchased from Bachem (Switzerland). [^{3}H]β-Endorphin, [^{3}H]dynorphin A and [^{3}H]α-neoendorphin (for synthesis see Houghten et al., 1980; Johnson et al., 1982; Houghten et al., 1983) had sp. act. of 45, 42 and 43 Ci/mmol, respectively. The tritiated peptides were purified just prior to use by high pressure liquid chromatography as described by Maysinger et al. (1982). [Tyrosyl-3,5-^{3}H] leu-enkephalin (43 Ci/mmol) was pur-chased from Amersham, U.K.

RESULTS

Experiments were conducted to evaluate the ad-vantage of the affinity chromatography gel (antibody 3-E7 linked to Sepharose) to separate opioid peptides from PBS. The peptides applied were β-endorphin, dynorphin A and α-neo-endorphin: in each case, 2 pmol in 10 ml PBS were used. An internal standard of 5 × 10^4 cpm (1 pmol) of the tritiated peptide was added to the corresponding sample. Figure 1 sum-marizes the data acquired from these experiments. The left panel depicts the results obtained with β-endorphin. The PBS wash (flow rate 0.5 ml/min)
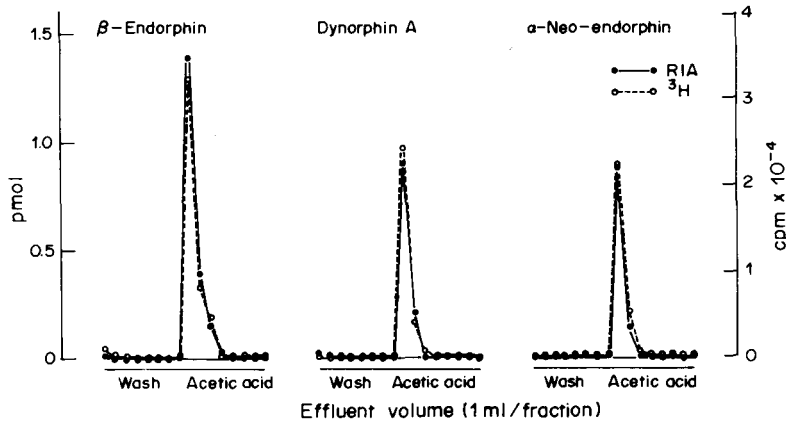
Fig. 1. Immunoaffinity chromatography of β-endorphin, dynorphin A and α-neo-endorphin. Each sample contained an internal standard of the respective tritiated peptide (5×10^4 cpm). The peptides were administered to the column in a volume of 10 ml PBS, and the gel was washed with 8 ml PBS. Elution was conducted with 2.5 M acetic acid. Each fraction collected was examined for the peptide concentration (radioimmunoassay, pmol/tube) and for tritium activity (cpm/tube).

did not elute significant cold (measured by RIA) or tritiated material. Use of 2.5 M acetic acid eluted with the first ml 96% of the cold and 92% of the hot β-endorphin. Almost identical results were obtained for leu-enkephalin (data not shown). The other panels present the results obtained with dynorphin A and α-neo-endorphin, respectively. As with the data for β-endorphin, the opioid peptides were eluted with acetic acid (2.5 M) subsequent to the wash procedure. The recovery of dynorphin A was 55% as measured by RIA and 58% for the tritiated material. The values for α-neo-endorphin are 51 and 55%, respectively. The search for the remaining material revealed that roughly 50% of the respectively tritiated peptides were present in the PBS fraction which had been run through the column in the course of the loading procedure. The reconditioned gel was reemployed 2 and 4 weeks later and was found to have an unchanged capacity to bind the three opioids.

Further experiments were conducted with 10 times the amount of immunoaffinity gel in order to increase the recovery of dynorphin A and α-neo-endorphin. The elution profile revealed only a moderate augmentation of about 10% for each peptide.

The capacity of the standard affinity column (0.5×5.0 cm, 1 ml gel) to bind an opioid peptide was tested with α-neo-endorphin in two experiments. Increasing amounts of this peptide (2–5000 pmol), containing [^{3}H]α-neo-endorphin (each 5×10^4 cpm), were applied in 10 ml PBS samples. Apparently, up to 0.5 nmol is bound by 1 ml gel, while higher amounts exceed the capacity.

Further experiments were conducted to examine the ability of the affinity gel to bind α-neo-endorphin at different pH values. 2 pmol α-neo-endorphin were mixed with the tritiated peptide (5×10^4 cpm) and applied to the affinity column (1 ml gel). The recovery of the tritium activity eluted with acetic acid reveals that the gel retains the peptide equally well at pH 7 and 8. However, the recovery sharply declines at lower or higher pH-values. Almost identical experiments were obtained for β-endorphin. It appears, thus, that pH 7.4 may be ideal for peptide-loading of the affinity gel.

In order to utilize the immunoaffinity gel for the purification of an opioid peptide from biological material, 5×10^7 neuroblastoma cells (NG 108CC15) were examined for the presence of α-neo-endorphin, an opioid peptide known to occur in these hybrid cells (Schulz et al., 1983). [^{3}H]α-Neo-endorphin (7×10^4 cpm) was added to the cells prior the extraction procedure and served as an internal standard. The extract (10 ml, pH 7.4) was applied to the standard affinity column within 3 h at 4°C. Figure 2A displays the wash and elution profile of the immunoaffinity gel. The α-neo-endorphin-like immunoreactivity assayed in the eluate amounts to 1 pmol (corrected for tritium activity), the recovered tritium activity was 44%. The fractions containing the immuno- and tritium-activity were pooled, lyophilized and applied to a column (Sephadex G-50). The elution profile is given in Fig. 2B. Most of the α-neoendorphin-like activity and the tritiated material is found within fractions 53–62, which coincides
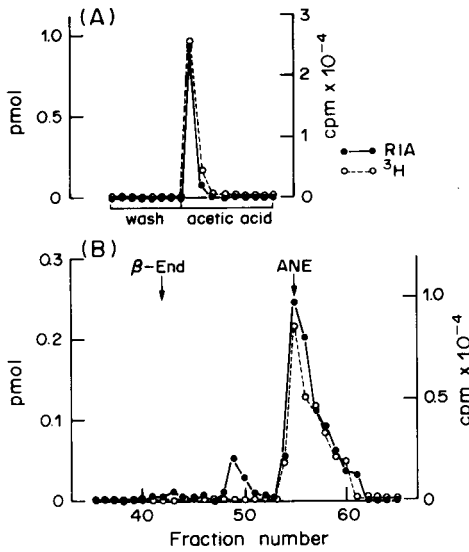
Fig. 2. α-Neo-endorphin purification of NG 108CC15 hybrid cell extract by immunoaffinity chromatography (panel A) and Sephadex G-50 gel chromatography. The material eluted from the affinity column was lyophilized and subjected to gel-chromatography (panel B). The samples were analyzed for [^{3}H]α-neo-endorphin and for α-neo-endorphin-like material by RIA. Synthetic β-endorphin (β-End) and α-neo-endorphin (ANE) were employed for calibration of the G-50 column.

with the elution profile of synthetic α-neo-endorphin. The tritium activity recovered in these fractions amounts to 39% and the α-neoendorphin-like immunoreactivity was 0.73 pmol. Thus, considering the recovery of the internal standard, an amount of 1.87 pmol per 5×10^7 hybrid cells was assayed. However, Fig. 2B also reveals the presence of material recognized by the α-neoendorphin antibody, but of a higher molecular weight than α-neo-endorphin. It is, thus, apparent that the α-neo-endorphin antibody recognizes material not identical with α-neo-endorphin, and this material was also recognized by the affinity column.

Further experiments were undertaken to examine the use of the immunoaffinity gel for the separation and concentration of opioid peptides from larger volumes. Thus, 2 pmol of β-endorphin, dynorphin A and α-neo-endorphin, respectively, were mixed with 5×10^4 cpm of the respective tritiated peptides and added to 200 ml cold tissue culture medium (RPMI 1640, containing 6% fetal calf serum, pH 7.4). The culture medium was heated to 90°C (5 min), centrifuged (3000 g) and applied to affinity columns (4°C) over 12 h. Subsequently, each column was washed

Table 1. Per cent recovery of opioid peptides in RPMI164 culture medium*

Compound	Recovery (%)	
	^{3}H-Material	RIA
β-Endorphin	79	84
Dynorphin A	41	43
α-Neo-endorphin	47	44

*Each peptide given to 200 ml RPMI1640, flow rate 0.28 ml/min, 4°C.

and eluted as described. Table 1 reflects the recovery of the three peptides investigated, both in terms of tritium activity and RIA. About 80% was recovered for β-endorphin and about 40% for dynorphin A and α-neo-endorphin, respectively. The recovered tritium activity and the corresponding RIA activity agree quite well for the three peptides tested.

The high recovery of synthetic β-endorphin at different experimental conditions by means of immunoaffinity chromatography encouraged the search for this opioid peptide in NG108CC15 cells. Nine batches of Meloy-cells were subjected to β-endorphin immunoreactivity. Table 2 depicts the immunoreactivity measured in "purified extract". Apparently, at least 3.5×10^8 cells were required to detect activity. The concentrations of β-endorphin immunoreactivity in 6 batches ranged from 3.7 to 16.8 fmol/10^8 cells. Batches H and I were subjected to dynorphin A and α-neo-endorphin RIAs. Batch H contained 84 fmol dynorphin A and 402 fmol α-neo-endorphin per 10^8 cells. The corresponding values for batch I are 108 and 45 fmol. In order to avoid any contamination of the cells with exogenous opioid peptides, the samples were not mixed with an internal ^{3}H-standard. The values were corrected according to the degree of recovery specified above, that is, 90% for β-endorphin, and 50% for dynorphin A and α-neo-endorphin. The combined "purified extract" of batches D to I was further subject to HPLC.

Table 2. Immunoreactive β-endorphin in affinity "purified extract" of NG 108CC15 cells

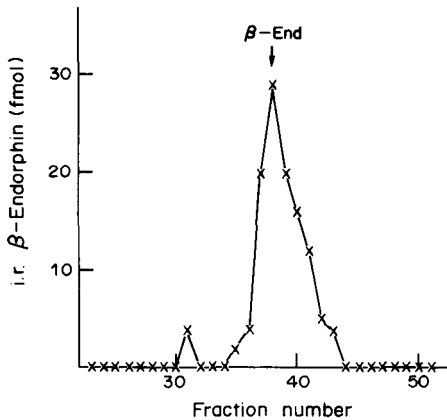| Batch	Meloy-cells ($\times 10^8$)	Immunoreactive β-endorphin	
		fmol	fmol/10^8 cells
A	0.05	<4*	—
B	0.5	<4	—
C	0.5	<4	—
D	3.5	48	13.7
E	3.5	21	6.0
F	3.5	13	3.7
G	3.5	18	5.1
H	14.0	165	11.7
I	14.0	235	16.8

*Detection limit 2 fmol/tube.

Fig. 3. HPLC analysis of immunoreactive (i.r.) β-endorphin in "purified extract" of NG108CC15 cells. Arrow indicates the elution volume of synthetic β-endorphin.

Figure 3 clearly demonstrates the presence of immunoreactive β-endorphin with a retention time identical to that of the respective synthetic peptide. Virtually identical results were obtained in a second run of the same "purified extract" under identical experimental conditions.

In an extension of the analysis of β-endorphin immunoreactivity "purified extract" of 2×10^9 MPI-cells were subjected to gel-chromatography (Sephadex G-50 superfine). The elution profile of immunoreactive β-endorphin reveals peak concentrations where synthetic β-endorphin elutes.

DISCUSSION

The major finding reported here is the detection of β-endorphin immunoreactivity in NG108CC15 hybrid cells. This finding was facilitated by an immunoaffinity purification procedure, employing the monoclonal β-endorphin antibody 3-E7.

The evidence for β-endorphin-like material in NG108CC15 cells rests on several experimental approaches, that is, immunoaffinity chromatography, HPLC and gel-filtration analysis combined with a highly sensitive β-endorphin RIA. This finding extends, thus, reports regarding the presence of enkephalins and dynorphin A/α-neo-endorphin in these hybrid cells (Glaser et al., 1980, 1982; Schulz et al., 1983). Since it has been proposed that NG108CC15 cells contain the precursors of the enkephalins and of dynorphin A/α-neo-endorphin (Schulz et al., 1983), we suggest that these cells also contain POMC, the

precursor of β-endorphin (Kakidani et al., 1979). It is assumed that these hybrid cells own the ability to generate each of the three families of opioid peptides (Höllt, 1983).

Previous investigations of the absolute concentrations of opioid peptides revealed considerable differences between various batches of the hybrid cells (Glaser et al., 1982; Schulz et al., 1983). With respect to β-endorphin immunoreactivity reported here, variations in concentrations were found too, although less pronounced. In general, however, the amount of β-endorphin activity in an individual batch was at least 10-fold less as compared to dynorphin A and α-neo-endorphin. Comparing the β-endorphin values with α-neo-endorphin of a different batch of hybrid cells more than 200-fold lower concentration was determined. These data demonstrate the difficulty in relating a particular amount of these opioids to the number of the hybrid cells in order to determine reliable concentrations. Factors affecting the opioid concentrations may be, e.g. differential processing of the precursors or the density of the cells under particular culture conditions (Glaser et al., 1982). Although the β-endorphin concentration found in the hybrid cells is low, there is no reason to discount these amounts. Whether the turn-over of this peptide or other regulatory mechanisms of the cell are responsible for these levels remains in need of clarification.

The application of immunoaffinity chromatography is considered a potentially efficient technique for the separation of opioid peptides from the "crude extract", since the antibody 3-E7 not only concentrated β-endorphin-immunoreactivity from a volume up to 200 ml, but also fails to recognize N-terminal extended opioid peptides, such as N-acetyl-β-endorphin or β-lipotropin (Gramsch et al., 1983; Meo et al., 1983). Use of the immunoaffinity gel for the purification of β-endorphin yielded an 80–90% recovery under various experimental conditions. This observation underlines the previously reported high avidity of the 3-E7 antibody for β-endorphin (Gramsch et al., 1983). Since this antibody completely cross-reacted with a number of opioid peptides, including met- and leu-enkephalin, BAM 22 and α-endorphin, a similarly effective purification of such peptides may be expected. Furthermore, the immunoaffinity procedure proved acceptable for the isolation of dynorphin A and α-neoendorphin. These peptides are recognized by the monoclonal antibody, although the avidity to these is lower than for β-endorphin (Gramsch et al., 1983; Meo et al., 1983). It may be, thus, not sur-

prising that about 50% of these peptides applied to the immunoaffinity gel ran through during the loading procedure. However, the reduced recovery was by no means due to an exhausted capacity, which is rather stable up to 0.5 nmol per ml gel.

In conclusion, the presence of immunoreactive β-endorphin in NG108CC15 cells complements previous findings regarding the presence of enkephalins and dynorphin A/α-neo-endorphin. The detection of β-endorphin-like material was facilitated by use of the monoclonal antibody 3-E7 in an immunoaffinity chromatography system. The often regarded disadvantage of antibodies displaying cross-reactivities to different opioid peptides emerges as a great advantage upon use of the antibody for an immunoaffinity procedure as a first step purification. The affinity chromatography described here appears, therefore, favourable as compared to other purification techniques, including the immunoaffinity systems as yet reported (Yamaguchi *et al.*, 1980; Shiomi and Akil, 1982; Gramsch *et al.*, 1979), as it does not purify only a single opioid. In fact, application of the herein described immunoaffinity procedure in the search for, as yet, unknown opioid peptides from biological material proved essential for the detection of Peptide F_{1-26}-amide, a novel C-terminally amidated opioid peptide (Liebisch and Seizinger, 1984).

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