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Melatonin-like immunoreactivity in the pineal gland of the cow: an immunohistochemical study

J.C. Carvajal, M.B. Gómez Esteban, S. Carbajo and L. Muñoz-Barragán

Department of Human Anatomy and Histology, Faculty of Medicine, University of Salamanca, Spain

Summary. With a view to checking the presence of melatonin in the pineal gland of the cow, in the present work we used six adult animals, ranging in age from one to six years, which were sacrificed at dawn. Sections of 6 µm thickness of Bouin-fixed and paraffin-embedded pineal glands were incubated in an anti-melatonin serum, which was provided by the Institute for Molecular and Cellular Recognition, Gunma University, Maebshi, Japan. After incubation and successive washings in PBS, some of the sections were treated with the avidin-biotin-peroxidase complex (ABC) technique using antisera from Sigma, and developed with the method of Graham and Karnovsky (which employs 3,3'-diaminobenzidine and H_2O_2 as developer). Other sections were incubated in a goat-anti-rabbit IgG (H+L) bound to fluorochrome Cy5 for immunofluorescence studies. An intense reaction for melatonin was observed in the cytoplasm but not in the nucleus of melatonin secreting pinealocytes located in peripheral and intermediate zones of the pineal gland. Immunoabsorption of the antimelatonin primary antibody with melatonin at a dilution of 10 mM per 0.1 ml of serum prevented the reaction, as happened when any of the antisera used in the procedure were used. Immunoabsorption of anti-melatonin serum with different amounts of bovine albumin (ranging between 1/5 to 1/50) failed to inhibit the immunoreactivity. When a bovine anti-albumin antibody was employed, working with the above methods, no immunoreaction was detected. Our data suggest that the pinealocytes of cows sacrificed at dawn contain immunoreactive melatonin.

Key words: Cow, Melatonin, Pinealocytes, Immunocytochemical

Offprint requests to: Prof. Luciano Muñoz Barragán, Departamento de Anatomía e Histología Humanas, Facultad de Medicina, Universidad de Salamanca, Avenida de Alfonso X el Sabio s/n, 37007 Salamanca, Spain. Fax: 34 923 294687. e-mail: lmunoz@usal.es

Introduction

Since melatonin was isolated by Lerner et al. in 1958, it has been considered to be the main hormone produced by the pineal gland, although its presence has been detected in other tissues such as the retina (Pang and Allen, 1986; Cahill et al., 1991), the intestine (Rhaikhlin et al., 1975; Quay and Ma, 1976; Bubenik et al., 1977; Holloway et al., 1980), and the Harderian gland (Vivien-Roels et al., 1981). The synthesis and release of melatonin generally occur during the darkness (Quay, 1963; Wurtman and Axelrod, 1965; Brownstein, 1975), although significant peaks have been observed at the end of the scotophase both in humans (Follenius et al., 1995) and in sheep (Redondo et al., 2003). The presence of melatonin in the pineal gland has been detected by radioimmunoassay (Wurzburger et al., 1976; Kennaway et al., 1977) and the hormone has also been detected in the cytoplasm of the pinealocytes of animals of different species, using immunocytochemical and immunofluorescence techniques (Vivien-Roels et al., 1981; Tillet et al., 1989, among others). More recently, other authors have claimed to have demonstrated the presence of immunoreactivity against melatonin inside the nucleus of pinealocytes (Menéndez-Peláez et al., 1993). Nevertheless, regarding the localisation of melatonin immunocytochemical techniques do not always afford positive or reproducible results, and some authors have employed antimelatonin antisera raised against complexes of melatonin with certain proteins, such as albumin and thyroglobulin (Vivien-Roels et al., 1981; Tillet et al., 1986, 1989). Moreover, it is well known that albumin can be detected in different cells, among which neurons and astrocytes should be mentioned (Dziegielewska et al., 1981; Uriel et al., 1983; Mollgard and Jacobsen, 1984; Medina and Tabernero, 2002; Tabernero et al., 2002a,b). Thus, the aim of the present work was to employ the avidin-biotin-peroxidase complex (ABC) and immunofluorescence techniques to detect the presence of melatonin-like material in the pineal glands of cows of different ages sacrificed at the end of the scotophase.

Materials and methods

The pineal glands of cows with ages ranging between 1 and 6 years were obtained from animals sacrificed at dawn. Immediately after extraction, the glands were fixed in Bouin solution, embedded in paraffin and cut in sections of 6 µm thickness.

After the sections had been deparaffinized in xylol, they were hydrated by passing them through a descending alcohol series to the PBS buffer (0.01 M, pH 7.4). All sections were preincubated for 30 min in normal goat serum at a dilution of 1:30 in 0.01 PBS buffer, pH 7.4, in order to prevent non-specific protein binding. Without washing, although after shaking off the drop of normal serum, the sections were then incubated in rabbit anti-melatonin serum at a dilution of 1/3000 in PBS buffer for 24 hours at room temperature. The anti-melatonin antiserum was provided by the Institute for Molecular and Cellular Regulation of Gunma University, Maebshi, Japan. Then, the avidin-biotin-peroxidase complex method (ABC) was applied (Hsu et al., 1981), following the method of Graham and Karnovsky (1966), which uses 3,3'-diaminobenzidine (Sigma) and hydrogen peroxide. Secondary antibodies were obtained from Sigma and were used at a dilution of 1/50 in 0.01 M PBS buffer, pH 7.4, over 30 min at room temperature.

Other sections were incubated in normal goat serum and then in the same primary antiserum as that described above. After a wash in PBS, they were treated with goat-anti-rabbit IgG (H+L) bound to fluorochrome Cy5 (from Jackson Immunoresearch) at a dilution of 1/100 for 30 min for immunofluorescence studies. With the same procedures, other sections were incubated in goat anti-bovine serum albumin (provided by Biomeda) at different dilutions (1/500, 1/1000, 1/2000, 1/4000 and 1/8000) to rule out or detect the presence of albumin inside the pinealocytes. Further controls were as follows: a) absorption of the anti-melatonin antiserum with melatonin (Sigma) at a dilution of 10 mM per 0.1 ml of primary antibody, or with bovine albumin at dilutions of 1/5, 1/15 and 1/50 per 1.0 ml of primary antiserum; and b) alternately excluding each of the reagents from the procedure.

Results

An intense melatonin-like immunoreactivity was observed in the cytoplasm of the pinealocytes from cows of all the ages used in the present study when the sections were incubated in anti-melatonin antiserum followed by the ABC reaction (Fig. 1). This reaction was very pronounced in peripheral zones of the gland and other intermediate areas of the gland in pinealocytes located in the proximity of calcifications, whereas in a large part of the glands the reaction was scarce or absent. The positive reaction was visualised as small intracytoplasmic accumulations and in no case was any melatonin-like immunoreactivity observed inside the nucleus of the cells, although small immunoreactive granules appeared and apparently appended to the nuclear envelope. Melatonin-like immunoreactive material was also found in cytoplasmic processes located around some capillaries. No reaction was observed in the profuse network of glial cells present in the pineal glands of the animals.

In sections incubated with primary antiserum previously absorbed with melatonin, no type of reaction was observed. This was also the case when any of the antisera in the reaction procedure were omitted. Additionally, the immunoabsorption of anti-melatonin antiserum with bovine albumin at dilutions of 1/5, 1/15 ad 1/50 in PBS did not alter the melatonin-like immunoreactivity present in the pinealocytes.

In sections incubated with anti-melatonin antiserum followed by treatment with secondary antiserum, the reaction was diffuse throughout the cell cytoplasm, although no immunoreactivity was observed in the cell nucleus or in any other structure comprising the gland, with the exception of the walls of some pineal vessels (Fig. 2). Likewise, immunoabsorption of the primary antiserum with melatonin failed to elicit any type of reaction.

Finally, the presence or not of albumin in the cytoplasm of bovine pinealocytes was investigated by incubating pineal gland sections in bovine anti-albumin antiserum, using both the ABC technique and immunofluorescence. Using either of these, here it was not possible to visualise the presence of immunoreactivity either in the cytoplasm or in the cell nucleus at any of the dilutions used (1/5, 1/15 or 1/50) (Fig. 3).

Discussion

Since Arendt et al. (1975), Lemaitre and Hartman (1980) and Grota et al. (1983) described a new technique for the production of antibodies, the presence of melatonin in the pineal gland of different animal species has been demonstrated with radioimmunoassay and immunocytochemistry. Thus, Tillet et al. (1986) observed the presence of melatonin in the pineal gland of sheep, while Tillet et al. (1989) reported the presence of melatonin in the pineal gland of mink. Using immunofluorescence or immunoperoxidase techniques, Vivien-Roels et al. (1981) reported the presence of melatonin in the pineal gland, the retina, and in the Harderian gland of different animal species, corroborating previous findings (Cardinali and Rosner, 1971; Raikhlin et al., 1975; Bubenik et al., 1976, 1977; Pang et al., 1976; Quay and Ma, 1976; Gern and Ralph, 1979; Wainwright, 1979; Holloway et al. 1980; Pévet et al., 1980). The binding of melatonin to proteins, among them albumin, for the collection of primary antiserum casts doubt on whether the immunoreactive material observed is in fact melatonin since no work has been published addressing the possibility that the immunoreactive material does in fact correspond to the protein used together with melatonin to immunise the

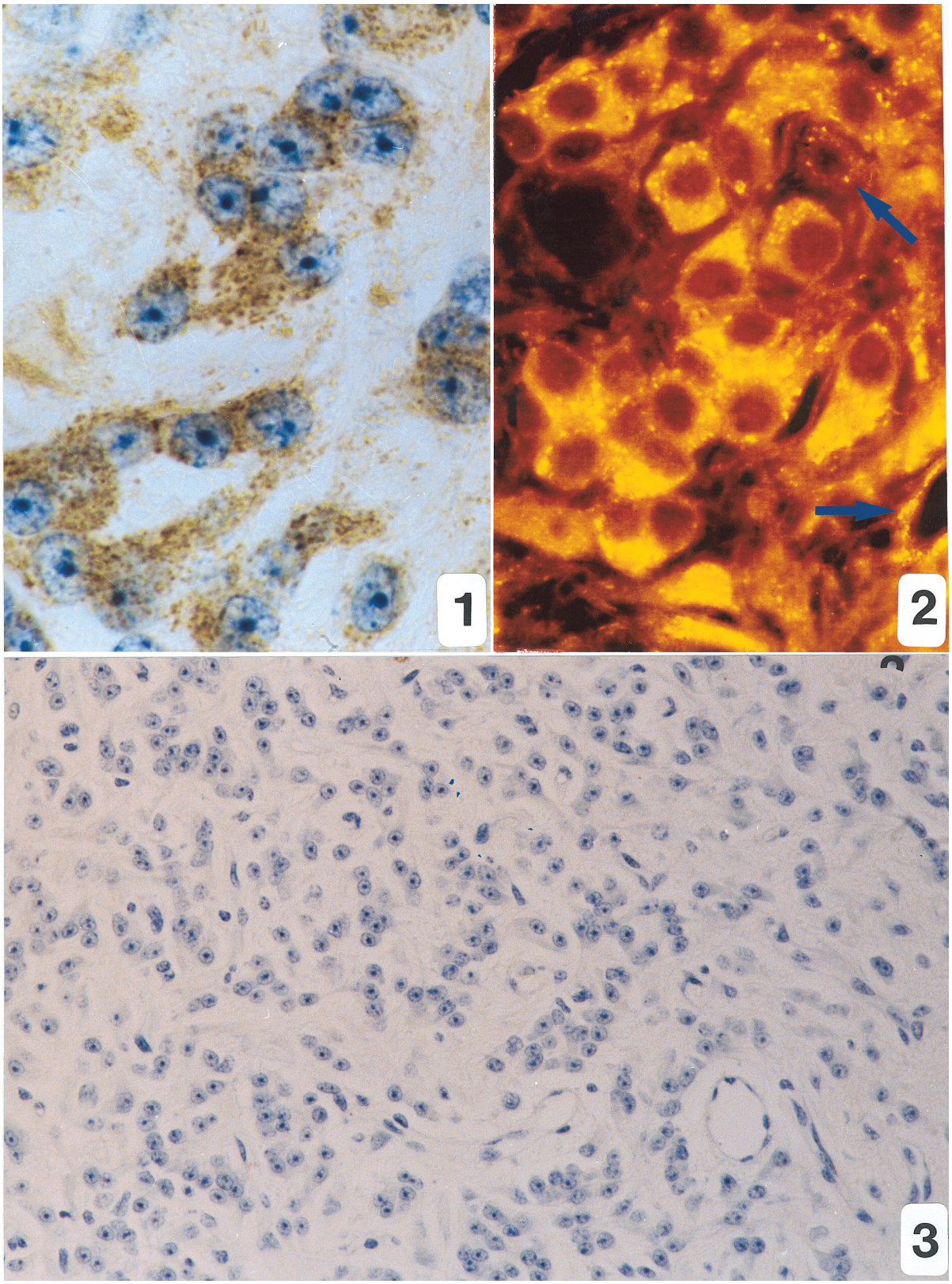

Fig. 1. Light micrograph of a portion of the cow pineal gland incubated with anti-melatonin antiserum (dilution 1/3000) according to the ABC technique and developed with 3,3'-diamino-benzidine and H_2O_2. Note the intense reaction located in the form of ccumulations within the cell cytoplasm of all the pinealocytes. x 1200

Fig. 2. Light micrograph of a section of the cow pineal gland incubated with anti-melatonin antiserum (dilution 1/3000) and second antiserum bound to fluorochrome Cy5. Note the intense fluorescence localised in ccumulations and diffused throughout the cell cytoplasm and in cytoplasmic processes located in the immediate vicinity of some blood vessels (arrow). x 1250

Fig. 3. Light micrograph of a section of the cow pineal gland incubated with anti-albumin antiserum (dilution 1/2000) following the ABC method and developed with 3,3'-diaminobenzidine. Note the total absence of immunoreactivity. x 250

animals.

In the present work, in which we investigated the presence of melatonin-like immunoreactivity in the pineal gland of cows, the anti-melatonin antiserum used was raised in rabbits against the melatonin-valeric acid-bovine serum albumin complex. It should first be noted that the immunoreactivity always appeared in the cytoplasm and never in the cell nucleus, even though the small immunoreactive granules appearing apparently appended to the nuclear envelope. In this regard, it should be stressed that we were working with 6 µm-thick sections, so it would be very difficult to determine whether the immunoreactivity observed in this structure (nuclear envelope) was outside, inside, or between the two membranes of this structure. This location is similar to those reported by Vivien-Roels et al. (1981), Falcon et al. (1981), and Tillet et al. (1989) in photoreceptor cells and pinealocytes. However, it contrasts with the findings of Mennenga et al. (1991), Menéndez-Peláez et al. (1993) and Menéndez-Peláez and Reiter (1993), who attempted to demonstrate the presence of melatonin immunoreactivity within the cell nucleus. Here, we were unable to corroborate this with any of the techniques employed, even though we were working with 1:200 dilutions of primary antiserum. It should be recalled that Menéndez-Peláez and Reiter (1993) advanced three reasons to rebut the findings of Bubenik et al. (1976, 1978) and Freund et al. (1977). The first was that the latter authors used anti-melatonin antisera at dilutions of 1:8 or 1:10. The second was the poor fixing of the glands from being immersed in ethanol and acetone for only a few seconds, and the third was the possibility that the antisera used could have shown an important cross-reaction with substances other than melatonin, such as 6-hydroxymelatonin, 5-methoxymelatonin, and others. This did not seem to be the case in our assays since the pieces were fixed in Bouin solution and the antiserum was used at very high dilutions (1:3000). Additionally, the cross-reactivity is very low (0.65% for 6-hydroxymelatonin, 0.098% for N-acetylserotonin, and <0.025 for methoxytryptophol), whereas serotonin, tryptophan, and other indole compounds show no cross reaction, at least according to the manufacturers of the antiserum used here and the experience of several other authors who have used the same antiserum previously (Sánchez-Vázquez et al., 1997; Iigo et al., 1997, 2003; Murakami et al., 1997, 2001; Nakahara et al., 1997, 2002; Hayashi et al., 1999).

Since immunoabsorption of the primary antiserum with melatonin led to the disappearance of immunoreactivity, while when it was carried out with albumin the immunoreactivity was not modified, our findings suggest that the immunoreactive material demonstrated was indeed melatonin. This is supported by the fact that in no case did we observe the presence of albumin-like immunoreactive material in the cytoplasm of the pinealocytes when working with bovine anti-albumin antisera and following the same procedure. We insisted on ruling out the possible existence of a cross-reaction with albumin for two reasons. The first was because albumin is a protein that is very abundant in cells and tissues, as well as in blood plasma. In this sense, many authors have demonstrated the presence of this protein in different parts of the central nervous system and the cerebrospinal fluid (Trojan and Uriel, 1979; Dziegielewska et al., 1981; Uriel et al., 1983; Mollgard and Jacobsen, 1984), while Medina and Tabernero (2002) and Tabernero et al. (2002a,b) have demonstrated the presence of albumin in neurons and astrocytes, where it would be internalised in such cells by receptor-mediated endocytosis. It is known that there are six fatty acid binding sites in the albumin molecule (Spector and Fletcher,, 1978) and it has been proposed that during brain development the role of albumin could be related to fatty acid transport (Calvo et al, 1998). In view of these results, the second reason why we wished to rule out a cross-reaction with albumin was the existence in the pineal gland of a large population of melatonin-secreting cells, which derive phylogenetically from photoreceptor cells, together with a considerable population of glial cells among which astrocytes are abundant. In our studies, working with an antialbumin antiserum we failed to detect albumin-like immunoreactivity in either melatonin-secreting cells or in astrocytes.

Additionally, the presence of immunoreactive melatonin-like material in animals sacrificed immediately after dawn seems to contradict the observations made over past decades in reference to the cyclic nature of the secretion of melatonin (Quay, 1963; Pang et al., 1980; Pévet et al., 1980), whose maximum levels are found both in the gland and in plasma during the first hours of the scotophase. In Spain, owing to BSE outbreaks it is currently very difficult to obtain fresh bovine material, so the material used here came from animals sacrificed in Chile at the start of the light period or end of the dark period. The fact that the material used was collected at this moment of the circadian rhythm could explain why the melatonin-like immunoreactivity did not appear throughout the gland, although Redondo et al. (2003) have reported the presence of significant levels of melatonin in the pineal glands of sheep sacrificed at 06:00 hours. The same result was reported by Follenius et al. (1995) in human beings and by Köhidai et al. (2002) in *Tetrahymena pyriformis* (a unicellular organism).

Detecting the presence of melatonin using immunohistochemical methods is not always easy and this is why such techniques have not become generalised in the study of melatonin in the pineal gland or other structures, at least from the experimental point of view. The failure of immunocytochemical techniques has been attributed to problems both in the manufacture of reliable antisera and to the fact that the synthesis and release of melatonin are very rapid and that this indolamine is not stored in the pineal gland but, instead, is released immediately after its synthesis through transmembrane diffusion, owing to the affinity of

melatonin for lipids and its liposolubility, which would favour its diffusion from the pinealocyte to the pericapillary space bound to a lipid carrier (Muñoz Barragán et al., 1988; Blázquez et al., 1992). However, an alternative explanation is that offered by Menéndez-Peláez et al. (1993) to the effect that the detection of melatonin by immunocytochemical methods at the level of the nucleus of pinealocytes or peripheral target cells would be a consequence of its ability to bind to some protein. This would favour the detection of the indolamine because it is stored in the cell cytoplasm, even though only for short periods of time, as reported by Köhidai et al. (2002), Simonneaux et al. (1989) and Pang et al. (1990). The existence of a residual pool of intracytoplasmic melatonin at the time of sacrifice of the animals could account for the presence of melatonin-like immunoreactivity detected by us in cow pinealocytes, confirming previous results employing different antimelatonin antisera in the pineal body of the turtle *Mauremys Caspica* (Muñoz Barragán et al., 1997).

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