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CELL CULTURE FROM TWO RED SEA BENTHIC INVERTEBRATES: A CASE STUDY ON THE SOFT OCTOCORALS *DENDRONEPHTHYA KLUNZINGERI* AND *ANTHELIA GLAUCA*

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ABSTRACT

Primmorphs were obtained from single cells of both coral species *Dendronephthya klunzingeri* and *Anthelia glauca* following incubation for three or four days during 2001. Both ultraviolet light (UVB; peak of emission of 320 nm) and visible light (wavelength between 400 and 520 nm with a maximum at 480 nm) were applied to the primmorphs as stressors. The following incubation probes have been isolated from D. klunzingeri and were used to monitor the expression of the respective genes in the homologous cell system; the heat-shock protein HSP90 (to monitor general stress responses), the histone H4cDNA (to monitor the cell cycle/proliferation) and the UVS-related protein (response to ultraviolet light) from *D. klunzingeri*. The data show that the steady state level of HSP90 expression is only upregulated at low level of exposure to UVB (30 J/cm^2), while at stronger irradiation the expression level decreases. The expression of the histone H4 gene is blocked following exposure to UVS. Interestingly, in the absence of UVB no transcripts of UVS-related protein can be visualized. However, after exposure of the primmorphs with 30 to 300 J/cm^2 a strong upregulation of the expression of the UVS-related gene is seen. The response of the primmorphs to visible light is distinct to that following exposure to UVB. The expression of the gene for histone H4 is significantly upregulated following exposure to UVB, while no expression of the gene UVS-related protein can be detected. It is concluded that, the primmorph system is useful to assess potential nature and anthropogenic disturbances on coral cells.

1. INTRODUCTION

Although UVB radiation has attenuated in seawater, harmful affects that can be measured at depths up to 20 m (Smith *et al*., 1992). Within the metazoan, the sessile species such as the Cnidaria and Porifera are the prime candidates for the adverse effects caused by UVB irradiation. It has been suggested that UV radiation is harmful for coral-reef epifauna (Jokiel, 1980; Ferrier-Bages *et al.*, 2007; Carpenter and Patterson, 2007) especially in clear coastal waters (Jerlov, 1950 and Smith and Baker, 1979).

The effects on corals are significant to depths (Jerlov, 1950). A decrease of skeletal growth and a parallel increase in larval production have been described as a result of increased solar UV (Jokiel and York, 1982). The photosynthesis of Zooxanthellae in corals is also severely affected by UVB (Halldahl, 1968). However, due to the fact that corals were present during the geological period characterised by thinner ozone layers in the stratosphere it can be postulated that they might have an inherent protection system against UVB irradiation. This notion has been supported in the past by the finding that corals produce a UV-absorbing ectodermal substance (Shibata, 1969) and have an adaptive UV protection system (Siebeck, 1981).

Visible light is attenuated in oceans with depth and accompanied by an alteration of the spectral composition (Jerlov, 1968 and Dustan, 1982). The waveband of maximum penetration is between 440 and 490 nm. This range parallels the absorption spectrum for zooxanthellae (Prezelin, 1981). To our knowledge no data on potential adverse effects of visible light on coral cells have been published.

In the present study a novel cell culture system is introduced to study the effect of UVB-and visible light on gene expression. The two Octocorallia *Dendronephthya klunzingeri* and *Anthelia glauca*, have been used. The primary coral cell cultures obtained underwent DNA synthesis. To our knowledge this has never been previously studied. In an earlier report cell cultures were established from larvae as well as from colony fragments of species belonging to Hexacorallia, Octocorallia and Hydozoa (Frank *et al*., 1994). In the present study molecular probes have been used, first to prove that true coral cells have been analysed, and second to monitor the response of the cells to UVB-and visible light. It has been established that, in addition of inducing DNA photoproducts, UVB also has effect on gene expression (reviewed in Herrlich *et al*., 1997). For example, the expression of collagenase is induced in human fibroblasts after irradiation with ultraviolet light (Stein *et al*., 1989). In contrast, the activity of a dephosphorylating enzyme is downregulated after UV exposure (Knebel *et al*., 1996).

The following molecular probes, cDNAs, have been isolated from *D. klunzingeri* and were introduced to monitor the homologous cell system. First: the heat-shock protein HSP90 which has previously been found to exhibit increased expression in *D. klunzingeri* after temperature shifts or after exposure to polychlorinated biphenyl compounds (Wiens *et al*., 1999). Second: the histone H4 cDNA from *D. klunzingeri* to monitor the cell cycle/proliferation (Reichheld *et al.,* 1998). Third, the UVS-related protein from *D. klunzingeri* which has a sequence similar to the UVS-2 protein from *Neurospora crassa* (Tomita *et al*., 1993) and to the RAD18 protein from *Saccharomyces cerevisiae* (Benit *et al*., 1992). UVS-2 is responsible for the removal of UV-induced photoproducts (Baker *et al*., 1991) and the RAD18 protein protects *S. cerevisiae* against UV damage (Game and Mortimer, 1974). The aim of this work, approach to that a novel cell culture system of soft octocorals *Dendronephthya klunzingeri* and *Anthelia glauca* are to study the effect of UVB and visible light on gene expression.

2. MATERIALS AND METHODS

2.1. Corals

Specimens of the soft corals *Dendronephthya klunzingeri* (Fig. 1.A) and *Anthelia glauca* were collected near Hurghada, Red Sea, Egypt.

2.1.1. Dissociation of cells and formation of primmorphs

A recently developed procedure was slightly modified and used to dissociate the coral cells and form primmorphs (Custodio *et*

al., 1998; Muller *et al*., 1999). In brief, tissue samples were submerged in CMFSW-E and cut into small cubes. Dissociation was performed in CMFSW-E in the presence of accutase (10 % [v/v]) for 1 hour at room temperature under constant rotation. The suspension was centrifuged (600 x g for 10 min) and the cells obtained were incubated in CMFSW-E for 1 h at room temperature. The cells were washed again by centrifugation (600 g for 10 min.) and transferred in CMFSW supplemented with antibiotics (100 IE of penicillin and 100 µg/ml of streptomycin). The suspension was adjusted to a density of approximately 10^6 cells/ml. After two days primmorphs, a special form of aggregate was formed. The primmorphs had a minimum diameter of 1 mm and an average diameter of 2 to 3 mm. Primmorphs were kept in seawater, supplemented with 1.3 g/l of RPMI 1640 medium (in solid form) and 3 g/l of Marine Broth 2216.

Primmorphs from *D. klunzingeri* or *A. glauca* were used for the experiments, after being maintained for four days in culture.

2.1.2. Exposure to Irradiation

Primmorphs were irradiated either with UVB or with visible light for UVB: the light source used in this study was a 20 W UVB lamp (UV-B TL 12RS Philips, Hamburg) at a peak of emission of 320 nm. Visible light: The Translux EC (Heraeus Kulzer, Wehrheim; Germany) halogen photocuring unit was used for these experiments. A wavelength between 400 and 520 nm with a maximum at 480 nm (Ernst *et al*., 1996) was used.

Primmorphs were exposed to 0 (controls) to 1,000 J/m^2. After irradiation the samples were left for 24 hours in the dark.

2.1.3. Extracts

To determine the levels of HSP90 protein coral extracts were obtained by grinding frozen tissue samples in three times their volume of phosphate-buffered saline, supplemented with 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride. After centrifugation the supernatant was collected and protein content was determined using the Lowry method (Lowry *et al*., 1951).

RNA was extracted from liquid-nitrogen crushed coral tissue with TRIzol Reagent (GibcoBRL) as recommended by the manufacturer. RNA was analysed as described by Preparation of the cDNA library from *D. klunzingeri* (Ausubel *et al*., 1995).

Total RNA was extracted from coral tissue and polyadenylated mRNA was isolated from total RNA (Pfeifer *et al*., 1993). cDNA was prepared by using the ZAP Express™ cDNA synthesis kit; instead of the ZAP Express vector, the Hybri-ZAPII vector containing the pAD-GAL4-plasmid (Stratagene) was used. The cDNA library of *D. klunzingeri* was prepared in Hybri ZAPII (Stratagene) and packaged in vitro using MaxPlax™ Packaging Extract (Epicentre Technologies). The resulting number of independent plaque forming units (pfu) of the library was approximately $10^{7;}$ the amplified library was stored at 4 °C.

2.1.4. Isolation of a cDNA encoding the putative HSP90 protein

The complete coral HS9DKEL cDNA was cloned by polymerase chain reaction (PCR from the *D. klunzingeri* cDNA library. The degenerate forward primer 5'-GATGCBGCYAAGAAYCA-3; (where Y = C/T; B = C, T, G) designed against the deduced nucleotide (nt) sequence of the conserved amino acid (aa) stretch ^{aa}629 to ^{aa}635 within the human SHP90 cDNA (HS86_HUMAN; accession number 72219 [Hoffmann and Hovemann (1988)] in conjunction with the pAD-GAL4 vector–specific reverse primer (5'-ACTCACTATAGGGCTCTAGA-3'). The PCR reaction was carried out using a GeneAmp 9600 thermal cycler (Perkin Elmer). An initial denaturation at 95 °C for 3 min was used, followed by 35 amplification cycles of 95 °C for 30 sec, 58 °C – 45 sec, 74

°C – 1.5 min. and a final extension step at 74 °C for 10 min. The reaction mixture of 50 µ/ included 20 pmoles of the degenerate primer and 10 pmoles of the vector–specific reverse primer pAD-GAL 4, 200 µM of each nucleotide, 1µ/ of the cDNA library, buffer and 2.5 units of Taq DNA polymerase. A DNA fragment of 603 bp was obtained. The clone was completed by PCR using primers corresponding to HS9DEKL which were designed to complete the 5'–terminus of the cDNA and using the vector specific primer (Ausubel *et al*., 1995). The longest insert obtained had a size of 2558 nt (excluding the poly (A) tail). The clone was termed HS9DEKL and was sequenced using an automatic DNA sequenator (Li-Cor 4200).

2.1.5. Isolation of a cDNA encoding the putative histone H4 protein

The complete cDNA sequence, encoding the putative *D. klunzingeri* histone H4 protein, H4DEKL was isolated by the same PCR strategy. The degenerate reverse primer 5'–TTIAGIGCA/GTAIACIACA/GTCCAT-3' (where I = inosine) in conjunction with the vector specific primer was used. The primer was designed against one conserved aa segment. The reaction conditions were the same as those for the isolation of HS9DEKL; an annealing temperature of 52 ^{o}C was chosen.

2.1.6. Isolation of the cDNA encoding the UVS-related protein

The partial sequence encoding the UVS-related protein, termed UVSRDEKL was cloned by PCR from the *D. klunzingeri* cDNA library. The degenerate forward primer 5' – AGA / GCAA / GATITTA / GGAGAAA / GCGICA-3' was used together with the vector-specific reverse primer; the annealing temperature was 53°C. The forward primer was designed against the conserved region found in the UVS-2 protein from *N. crassa* (P33288; Tomita *et al*., 1993), ^{aa}277 to ^{aa}286, and in the RAD18 protein from *Saccharomyces cerevisiae* (P10862; Benit *et al*., 1992), ^{aa}299 to ^{aa} 308. The partial sequence from *D. klunzingeri* is 851 nt long.

2.2. Sequence comparisons

The sequences were analyzed using computer programme (BLAST, 1997 and FASTA, 1997). Multiple alignment was performed with CLUSTAL W Ver. 1.6 (Thompson *et al*., 1994). A phylogenetic tree was constructed on the basis of aa sequence alignments by neighbour-joining, as implemented in the "Neighbour" programme from the PHYLIP package (Felsenstein, 1993) The distance matrix was calculated using the Dayhoff PAM matrix model as described by Dayhoff *et al*. (1978). The degree support for internal branches was further assessed by bootstrapping (Felsenstein, 1993).

2.2.1. Northern blot

RNA was extracted from liquid-nitrogen crusted coral tissue with TRIzoI Reagent (GibcoBRL) as recommended by the manufacturer. One µg of RNA was electrophoresed through formaldehyde/ agarose gel and blotted onto Hybond N^{+} membrane following the manufacture's (Amersham). Hybridization experiments were performed (i) with the prob HS9DEKL (603 pb segment), (ii) with the complete H4DEKL cDNA, (iii) with the UVSRDEKL fragment, all obtained from *D. Klunzingeri.* The probes were labelled with DIG-11-dUTP by the DIG DNA labelling kit. Hybridization was performed with the antisense DIG-labelled probes at 42°C over night using 50% formamide, containing 5xSSC, 2% blocking reagent, 7% [w/v] SDS and 0.1% [w/v] N-lauroylsarcosine, following the instructions of the manufacturer [Boehringer]. After washing DIG-labelled nucleic was detected with anti-DIG Fab fragments (conjugated to alkaline phosphatase) and visualized by

chemiluminescence technique using CDP, the chemiluminescence substrate alkaline phosphatase, according to the instructions of the manufacturer (Boehringer).

Quantification of the signals of Northern blots a chemiluminescence procedure was applied (Stanley and Kricka, 1990). CDP was used as substrate. The screen was scanned with the GC-525 Molecular Imager (Bio-Rad).

2.2.2. Western blotting

Extracts were subjected to electrophoresis in 7.5% polyacrylamide gels containing 0.1% NaDodSO4 as described by Laemmli (1970). For western-blotting experiments the proteins were electro-transferred to PVDF-Immobilon P membranes using a semi-dry blotting apparatus (Wiens *et al*., 1998). The membranes were incubated with monoclonal antibody against HSP90 (McAP-HSP90; 1: 500 dilution) for 1.5 hrs at room temperature, followed by incubation with peroxidase-conjugated anti-mouse IgG and CSPD; the blots were evaluated using a Model GS 525 Molecular Imager (Bio-Rad) (Stanley and Kricka, 1990).

2.2.3. Immunocytochemical detection of BrdU incorporation in cells of primmorphs

The BrdU (5-bromo-2'-deoxy-uridine)-labelling and detection assay (Gratzner, 1982) was used to demonstrate that the cells organized into the primmorphs regained the capacity to proliferate. The cells were incubated for 12 hrs in the presence of BrdU. Then the incorporation of BrdU into DNA was detected by an anti-BrdU monoclonal antibody. The nuclei of BrdU-positive cells, undergoing DNA synthesis, under this condition no staining was observed.

3. RESULTS AND DISCUSSION

3.1. PCR cloning and sequencing of the cDNA HS9DEKL

One degenerate oligonucleotide primer, corresponding to a nucleotide sequence of the region in human HSP90 cDNA, was used to identify the corresponding cDNA from the coral cDNA library, as described under Materials and Methods. The cDNA obtained, HS9DEKL, encodes the putative HS9_DEKL protein.

The largest cDNA obtained was 2558 nt in length (excluding the poly (A) tail; accession number Y15421) and contained a potential open reading frame (ORF), extending from nt 74 to 2272 and coding for 733 aa. At the 3'-terminus the cDNA has a poly (A) tail, with the polyadenylation signal AAUAAA (^{nt}2540 to ^{nt}2545). The putative AUG initiation site (^{nt} 74 to ^{nt} 76) displayed a strong consensus sequence A_3/T+4 (Kozak, 1991) and read AAAATGT (the putative translation initiation site is underlined). Northern blot analysis was performed with the sponge HS9DEKL -clone as a probe. One band of approximately 2.7 kb was obtained, confirming that the full length cDNA was isolated.

3.2. Amino acid sequence analysis and phylogenetic analysis of coral HSP90

The HS9DEKL cDNA encodes a 733 aa long putative HSP90, HS9_DEKL. The deduced M_r is 86,309 with a pI of 4.53 (PC/GENE, 1995). According to the instability index, the sponge HS9_DEKL has to be classified as an unstable molecule with a predicted half-life of 5.5 hr (PC/GENE [Physchem], 1995). The HSP90 protein family signature NKEIFL (^{aa}38 to ^{aa}43) is present (Lindquist and Craig, 1988). The stretch ^{aa}9 to ^{aa}16 shows characteristics of the component of HSP90s (Lees-Miller and Anderson, 1989a). Furthermore the repeats Glu-Lys (Moore *et al*., 1989) are found in the coral sequence within the segments ^{aa}222 to

[aa]282. The potential phosphorylation site at serine aa 229 for the casein kinase, which is also present in vertebrate HSP90 sequences (Lees-Miller and Anderson, 1989b), was found in the coral sequence.

Homology searches (BLAST and FASTA) with HS9_DEKL revealed highest similarity to metazoan HSP90 proteins. The coral protein sequence shares ≈ 75% of identical aa and ≈ 90% of similar aa with other corresponding molecules.

Earlier studies have revealed that the class of HSP90 proteins, including members of vertebrates and higher invertebrate phyla are phylogenetically closely related (Moore *et al*., 1989). As revealed from the present study we may conclude that within an unrooted tree constructed from HSP90 sequences from (i) Metazoa (including the vertebrates human, chicken and zebrafish sequences and the invertebrates *Drosophila melanogaster* and *Caenorhabditis elegans*, (ii) unicellular eukaryotes (*Trypanosoma cruzi* and *Plasmodium falciparum*), (iii) the slime mold *Dictyostelium discoideum*, (iv) Fungi (Ascomycota) and (v) viridiplantae (*Arabidopsis thaliana*), the coral HSP90 sequence from *D. klunzingeri* groups together with the invertebrates.

3.3. Cloning of the cDNA H4DEKL

The cDNA encoding the putative *D. klunzingeri* histone H4 H4_DEKL was cloned. The full length clone, H4DEKL, comprises 399 nt, and has an ORF of 309 nt (accession number Y15422). The deduced aa sequence, H4_DEKL, has a length of 103 aa and calculated M_r of 11,381. Based on the size of the H4 transcripts, 0.5 kb, the cDNA is considered to be complete. The consensus pattern characteristics for histone H4 proteins, Gly-Ala-Lys-Arg-His (Doenecke and Gallwitz, 1982; Ebralidse *et al*., 1988) is found in the *D. klunzingeri* sequence between [aa]15 and [aa]19.

Other comparisons (e.g. Ebralidse *et al*., 1988) have shown that the histone H4 polypeptides are highly conserved. The comparison demonstrates that the *D. klunzingeri* sequence is identical with the corresponding sequence from *Acropora formosa*, differs in one aa from the human sequence, in two residues from H4 from *C. elegans* and in three aa positions from H4 isolated from wheat. This analysis shows that the phylogenetic distance of the coral H4 molecule to the human sequence is shorter than that to one of the pseudocoelomata *C. elegans*.

3.4. Cloning of the cDNA *UVSRDEKL*

The UVS-related protein from *D. klunzingeri* was also isolated by PCR. The partial sequence encoding the UVS-related protein, UVSRDEKL (accession number Y15423), is 849 nt long and comprises an ORF for 266aa. Northern blot analysis revealed that the transcripts of the coral UVS-related protein have a size of 1.5 kb, reflecting a protein of approximately 480 aa in length. The coral UVS-related protein is slightly shorter than the 501 aa long UVS-2 protein from *N. crassa* (Tomita *et al*., 1993) and the 487 aa long RAD 18 protein from *S. cerevisiae* (Benit *et al*., 1992).

3.5. Primmorphs from *D. klunzingeri* as a model to determine the effect of UVB and visible light

Primmorphs obtained from dissociated cells of *D. klunzingeri* (Fig. 1.D) were used to determine the effect of increasing exposure to UVB and visible light (480 nm). Western blot experiments were performed to determine the effect of light on the level of HSP90 protein. The results revealed that after irradiation with a low level of UVB (30 J/cm^2) the primmorphs react with a strong (5.5 fold) increase of HSP90, compared with the controls. Upregulation was observed following an increase UVB irradiation to 100 J/m^2, while at levels above 100 J/m^2 no HSP90 was detected. In contrast, the

upregulation of HSP90 protein following exposure to visible light was less pronounced and reached a maximum at 300 J/m^2, with a 3.2-fold increase. Consistent loading of the gels with an equal amount of protein was verified in control experiments using polyclonal antibodies against actin.

Northern blot experiments were performed to determine the effect of UBV and visible light on the expression of the genes, encoding HSP90, histone H4 or the UVS-related protein. The "house-keeping" gene tubulin was used to confirm that the same amount of RNA was applied for each analysis.

It was obvious that the steady state level of HSP90 expression was only upregulated at low exposure to UVB (30 J/m^2) while stronger irradiation resulted in a decrease level. The expression of the histone H4 gene was not apparent following exposure to UVB; no signal was detected at the low exposure level of 300 J/m^2 or higher. Interestingly in the absence of UVB to transcripts of UVS-related protein could be visualized, while after treatment of the primmorphs with 30 to 300 J/m^2 a strong upregulation was seen in the expression of this gene.

The response of the primmorphs to visible light was distinct to the effect of UVB. The expression of HSP90 was generally strong and only at the high exposure level of 1'000 J/m^2 was no signal seen. The expression of the gene for histone H4 was significantly upregulated following exposure from 30 to 300 J/cm^2, while no expression of the gene for the UVS-related protein was observed.

3.6. Effect of light on the proliferation of coral cells

The BrdU-labelling and detection assay was applied to demonstrate whether the cells in the primmorphs underwent cell proliferation. The cells were incubated with BrdU; the incorporated BrdU into DNA was detected with anti-BrdU monoclonal antibody and used as a measure of proliferation. The nuclei of BrdU-positive cells undergoing DNA synthesis stained brown. The percentage of BrdU-positive cells in primmorphs obtained from cells of *D. klunzingeri* or of *A. glauca* was approximately 10%. However, following exposure to 1,000 J/m^2 of UVB no incorporation of BrdU into DNA was seen in the primmorphs.

In the present study it is shown that primmorphs can be formed from signal cells of the octocorals *D. klunzingeri* and *A. glauca.* The primmorphs of *D. klunzingeri* have been studied in most detail; the cells in these aggregate-like cell assemblies were found to undergo DNA synthesis. Under the culture conditions used approximately 10% of the cells in primmorphs proliferated.

The primmorphs from *D. klunzingeri* cells were used as a model system to analyze the effect of UVB and visible light on cnidarian cells in vitro. The following molecular probes were applied; (i) the cDNA encoding the HSP90 protein to estimate stress on cells in general, (ii) the cDNA for histone H4 to monitor cell cycle/proliferation and (iii) a cDNA fragment encoding the putative UVS-related protein as a first probe in corals for the determination of the effect of ultraviolet light.

The results revealed that primmorphs irradiated with UVB respond with an increased expression of HSP90 only if exposed to low levels of UVB. The expression studies using Northern blot analysis were confirmed by western blot experiments. UVB exposure causes a strong reduction of the expression of histone H4, even at low levels, indicating an inhibition of cell proliferation (Lukyanov and Chudakov, 2006; Harder *et al*., 2003). The adverse effect could also be monitored using an immunocytochemical detection assay by which the fraction of cells in the primmorphs undergoing DNA synthesis is determined. A sensitive marker of UVB light was found to be the cDNA fragment encoding the UVS-related protein. A strong upregulation of the

steady state level of the transcripts was quantified by Northern blot analysis.

In contrast to the studies with UVB, visible light causes an upregulation of the expression of the gene for HSP90 in a wide exposure range, between 20 and 1,000 J/m^2. No induction of the expression of the gene encoding the UVS-related protein was measured following exposure to visible light, while the expression of the histone H4 gene was transiently upregulated between exposures of 30 and 300 J/m^2.

The data reported in the present study prove for the first time that potential natural and anthropogenic disturbances on coral cells can be determined under *in vitro* conditions. Here the physical stress of light on coral cells has been studied. In the future, this approach will also be applied to estimate other stress factors such as the potential effect of polychlorinated hydrocarbons on gene expression using the gene encoding the targeting chaperone 14-3-3 (Wiens, *et al*., 1998). In addition, novel strategies may be used in environmental monitoring to estimate the health of a given coral reef. These can be performed in the primmorph *in vitro* cultures and include: identification of potential xenoestrogens using the testis enhancing gene as probe or assessment of the state of nutrition of corals uses the fructose-1-6-bisphosphotase cDNA as a probe. These molecular tools are available from *D. klunzingeri.*

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DISCLOSURE STATEMENT

A patent application has been filed with the title "Herstellung von Primmophe® aus dissoziierten zellen von Schwammen, Korallen und weiteren Invertebraten: Verfahren zur Kultivierung von Zellen von Schwammen und weiteren Invertebraten zur Produktion und Detektion von bioaktiven Substanzen, zur Detektion von Umweltgiften und zur Kultivierung dieser Tiere in Aquarien und im Freiland"; (AZ 198 24 384).

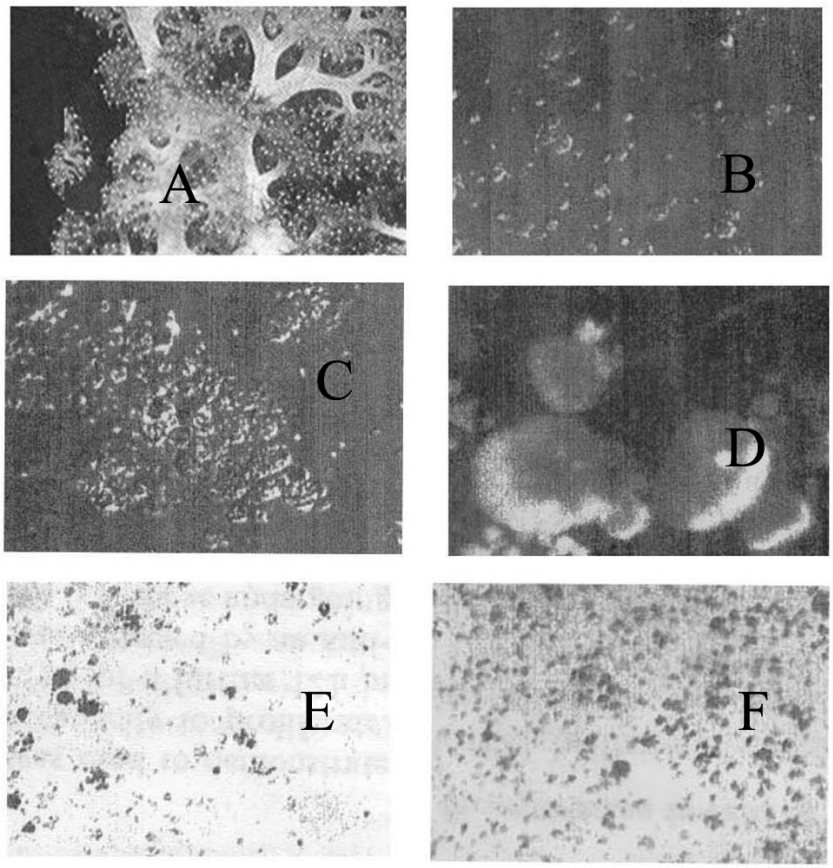

Fig. (1): Primmorphs formation from dissociated cells of the coral *Dendronephthya klunzingeri*. The octocoral *Dendronephthya klunzingeri* (A) was dissociated into single cells (B) using CMFSW-E, after transefer into seawater/antibiotic aggregates are formed (C) which finally resulted in the formation of primmorphs after an incubation period of 3-4 days (D). The dark brownish nuclei present in cells from untreated primmorphs are those which incorporated BrdU (E). Cells are treated with 1,000 Jm^{-2} of UVB

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